



Gillespie, James Innes (1977) Ionic blockage of the delayed potassium conductance of skeletal muscle fibres. PhD thesis.

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IONIC BLOCKAGE OF THE DELAYED POTASSIUM CONDUCTANCE
OF SKELETAL MUSCLE FIBRES

A thesis submitted to the University of Glasgow for
the Degree of Doctor of Philosophy in the Faculty
of Science

By

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October, 1977.

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SUMMARY

SUMMARY

- (1) The interactions of 4-aminopyridine with the delayed potassium conductance of skeletal muscle have been investigated. It is found that 4-aminopyridine (4-AP) is 10 times more potent than TEA ions in blocking the potassium conductance, and in addition 4-AP appears to block in a different way.
- (2) 4-AP is a weak base with a pK_a of 9.17. The distribution of such a weak base across the cell membrane and means for determining which class of the base is responsible for the biological activity are discussed.
- (3) When 4-AP is present on both sides of the membrane, the peak potassium conductance is reduced and there is an initial faster component to the timecourse of inactivation. When 4-AP is present on the outside of the fibre only the peak conductance is reduced ($K_{diss}=1.1mM$).
- (4) It is proposed that the cationic form of 4-AP is the active form and that it acts at two sites. One is accessible from the extracellular solution and is responsible for the reduction in peak current; and the other accessible from the intracellular solution and responsible for the initial faster component of inactivation.
- (5) After an initial delay the inactivation induced by 4-AP inside the fibre, $4-AP_i^+$, is found to decay exponentially at a rate which increases as the internal potential is made more positive.

- (6) The fraction of channels that become inactivated by 4-AP_i^+ in the course of a depolarizing step is found to increase as the internal potential is made more positive. In the voltage range -40mV to 0mV this fraction undergoes an e-fold increase every 12mV ; beyond $+10\text{mV}$ this fraction reaches a limit, the value of which depends only on the concentration of 4-AP .
- (7) The blocking actions of 4-AP outside the fibre, 4-AP_o^+ , can be separated into two components. One component is not influenced by the membrane electric field and the level of block depends only on the concentration of 4-AP . The second component is influenced by the membrane electric field and undergoes an e-fold change every 38mV , increasing as the internal potential is made negative.
- (8) Upon early repolarization of the membrane to -80mV this voltage dependent component of 4-AP_o^+ block proceeds exponentially with a time constant of 8 msec .
- (9) The blockage of the delayed channel by 4-AP_i^+ and 4-AP_o^+ is described in mathematical form; and the results discussed in terms of possible molecular structures and events within the channel.
- (10) The repolarizing phase of the action potential is slowed in the presence of 1mM 4-AP ($\text{pH } 7.2$). The resting potential, the rate of rise and overshoot of the action potential are little affected.
- (11) The inwardly rectifying component of the potassium conductance is reduced by 4-AP . This block is intensified in alkaline solution which suggests that the cationic form inside the cell is the active form.

- (12) The delayed potassium channel has also been studied in solutions of different pH. At pH 5.0, \bar{g}_K is reduced by 16.3% and the threshold shifted by 20.2 mV in the depolarizing direction. At pH 9.8, \bar{g}_K is reduced by 13% and the threshold shifted by 15mV in the depolarizing direction.
- (13) The delayed potassium current is abolished after treatment with 5mM 4-AP (pH 9.8). In these experiments, the transient current which remains is associated with the charging of the membrane capacity.
- (14) Asymmetries in the membrane capacity are observed, similar to those described by Schneider and Chandler (1973). These asymmetries are thought to be intra membrane currents associated with excitation contraction coupling. Formaldehyde, which interrupts excitation contraction coupling, reduces the asymmetry in the membrane capacity.

ACKNOWLEDGEMENTS

ACKNOWLEDGEMENTS

I would like to thank Professor O.F. Hutter and Professor I.A. Boyd for allowing me to carry out this work in the Institute of Physiology, University of Glasgow.

My most sincere and warmest thanks go to my supervisor Professor O.F. Hutter. Through his patient guidance, encouragement and example he has given me a greater awareness of many aspects of Physiological Science. For this I will be eternally grateful.

I would like to warmly thank all members of staff and students in the Institute of Physiology, Glasgow. In particular I would like to mention Dr. R.F. Burton, Dr. V. Moss and Mr. L.B. Spark for many hours of helpful and fruitful discussion.

I would also like to thank Mr. L.B. Spark for developing and maintaining the electronic equipment, and Mrs. J. Black and Mr. J. Wilson for technical assistance.

I am deeply indebted to Miss J. Brown and Miss N. McGuinness who worked wonders in typing the many drafts of this thesis and to Miss N. McGuinness for typing and preparing the final manuscript.

Lastly, I would like to thank my family, especially my wife, without whose encouragement and understanding the production of this thesis could have been many times more difficult.

J.I. GILLESPIE

I. INTRODUCTION

I; The Use of Chemical Agents as Tools in the Study of Excitable Membranes

In recent years our knowledge of events in excitable membranes has advanced through the study of how drugs and chemical agents exert their action. Information on the structure and the molecular events that occur within ionic channels, that was not available solely from voltage clamp analysis, can now be obtained. This thesis is concerned mainly with the delayed potassium channel of skeletal muscle and how clues to the molecular architecture of this channel may be obtained from studying the interactions of one such chemical agent, 4-aminopyridine. The usefulness of 4-aminopyridine as a tool in the study of other membrane events is also examined.

A large number of drugs and chemical agents that are now being used as membrane probes have been known for many years to be biologically active. However, their potential usefulness as tools in the study of excitable membranes was not fully realised until after the development of the voltage clamp technique and there was a basic description of the membrane currents. One of the more notable agents that has been used as a membrane probe is tetrodotoxin (TTX) (Narahashi, Moore & Scott, 1964; Narahashi, Anderson & Moore, 1966, 1967). TTX selectively blocks the sodium current in nerve, but only when applied to the outer surface, and leaves unaffected the delayed potassium current. Many other investigations into the mode of action of chemical agents have proved to be equally fruitful. Among the most interesting are, the tetraethylammonium ion and its derivatives; the local anaesthetics; the neurotoxins; and convulsive agents of which 4-aminopyridine is one.

The remainder of this introduction discusses (i) what information is already available on the molecular architecture of ionic channels, (ii) 4-aminopyridine: a review of its biological actions and its physical chemistry, and (iii) the potassium conductance of skeletal muscle.

Although this thesis is concerned with the potassium channel in the skeletal muscle membrane it is of interest to describe what is known about the structure of the sodium and potassium channels in nerve membranes from studies using chemical agents. The ideas first developed on nerve are similar to those that will be used later.

The Molecular Architecture of the Sodium and Potassium Channels in Nerve

The ionic channels in nerve membranes are generally considered to be aqueous pores with at least three distinguishable parts. At their outer and inner aspects they are approximately 8 \AA in diameter, and not particularly selective; somewhere within the channel there is a narrower section which is more selective, the "selectivity filter"; also located within the channel are voltage dependent gating mechanisms which determine whether the channel is open or closed. Some of the particular structural details of the sodium and potassium channels in nerve are outlined below.

The Sodium Channel

Experiments to determine the relative permeability of mono-valent ions, (Hille, 1973) have led to the suggestion that the selectivity filter has minimum dimensions $3 \times 5 \text{ \AA}$ and that it is an anionic site, probably carboxyl groups, of high field strength. The blocking action of the hydrogen ion at this site and the voltage dependence of the block suggests that the site is located three quarters of the way across the membrane from the outside (Hille, 1972; 1975; Woodhull, 1973).

The inner mouth of the channel is wider than the selectivity filter, and may be approximately 8 \AA in diameter, since it is able to accommodate the local anaesthetics lidocaine and dibucaine (Strichartz, 1973; Narahashi, 1974; Courtney, 1974). The outer mouth is different in structure, it does not accommodate the local anaesthetics but binds TTX tightly (Narahashi, 1974).

3

Binding studies with radioactively labelled TTX suggests that the density of sodium channels in the membrane of the squid axon is of the order of 10 per μ^2 . This corresponds to a single channel conductance of approximately 10^{-11} mho, which strongly supports the view that the channels are aqueous pores and not carrier molecules (Hille, 1975^b).

Internal perfusion of squid giant axons with chemical agents suggest that the gating molecules which determine whether the channel is open or closed are located near the inner surface of the membrane. The inactivation mechanism can be selectively digested away by internal perfusion with pronase (Armstrong, Bezanilla & Rojas, 1973), as a result the sodium currents activate normally but fail to inactivate with time. Such experiments also provide support for the assumption of Hodgkin & Huxley (1952d) that the activation and inactivation mechanisms are not inter-dependent (Armstrong, 1975a). However to the contrary, external application of centruroides toxin to frog node selectively modifies the activation gate (Cahalan, 1974); and condylactis toxin (Narahashi, Moore & Shapiro, 1969) and *Lyturus* toxin (Koppenhofer & Schmidt, 1968) drastically slow inactivation.

The Potassium Channel

One important difference between potassium and sodium channels is that potassium ions appear to interact with each other within the channel, (Hodgkin & Keynes, 1955; Armstrong, 1975a; Hille, 1975a). This has led to the suggestion that the channel is like a long narrow tunnel.

The permeability of the channel to small cations suggests that the narrow portion has a diameter of approximately 3 \AA (Bezanilla & Armstrong, 1972; Hille, 1968, 1973). The inner mouth may be slightly larger approximately 8 \AA and can accommodate large blocking molecules like TEA and its long chain derivatives, (Armstrong, 1975a & b; Armstrong & Hille, 1972). Associated with this inner area is a strongly hydrophobic site which binds C_9 more tightly than TEA, (Armstrong & Hille, 1972). The structure of the outer asp

is different in different tissues; in squid axon, this portion does not accommodate TEA (Armstrong & Binstock, 1965; Armstrong, 1975a), whilst in frog node of Ranvier the channel can be blocked by TEA outside the fibre (Koppenhöfer, 1967; Hille, 1967). The activation gate, of the potassium channel is located near the inner surface of the membrane. (Armstrong, 1975a & b; Armstrong & Hille, 1972), since the block induced by C_9 and TEA internally must wait for the gate to open.

A value of 2×10^{-12} mho has been calculated for the conductance of a single potassium channel from measurement of the rate of block by the C_9 ion (Armstrong, 1969, 1975a); although this is probably an underestimate this gives a channel density of between 10 - 100 per μ^2 .

I:ii The Aminopyridines

Pharmacological observations

The earliest observations on the biological actions of the aminopyridines were published by Dohrn (1925) and Dingemanse & Wibaut (1928). After injecting 2-aminopyridine (2-AP) subcutaneously or intraperitoneally into frog, rats or mice the animals developed tremours that were quickly followed by convulsions and death. Similar fatal convulsions were observed by Reinwein (1930) who injected the pyridinic fraction of yeast nucleic acid into dogs. Charonnat, Lechat & Chareton (1953 a & b) also found that the sometimes fatal convulsions caused by intravenous injection of thiamine were due to the pyridinic part of that molecule, and that a similar effect could be obtained using 4-AP, 2-AP or 3-AP. Von Haxthausen (1955a) carried out basic pharmacological studies on each isomer. He found that the aminopyridines were not only powerful central nervous stimulants, but also caused a marked increase in blood pressure. Moreover, they possessed analgesic activity approximately 70% that of morphine and showed slight local anaesthetic properties.

Another independent line of investigation which led to a study of the aminopyridines began with Smirk (1941) who discovered the pressor action of S-methylisothio-urea. The mode of action of this compound was of interest because it appeared to be radically different from the sympathomimetic amines. The pressor response of S-methylisothio-urea remained in animals rendered insensitive to the actions of ephedrine and amphetamine; neither was the response antagonised by adrenergic blocking drugs or by drugs which deplete the tissues of catecholamines (Fastier, 1962). In early investigations into this property of S-methylisothio-urea Fastier & Smirk (1947), Fastier (1948) and Fastier & Reid (1952) examined the activity of a range of structurally related compounds. One group of compounds which resembled S-methylisothio-uronium ions in their pharmacological actions were the amidines. The general

structure of the amidines is similar to the thio-ureas and is shown in Fig. 1; the sulphur atom in thio-urea is

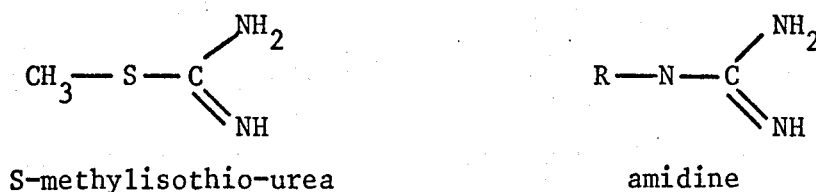


FIG.1

replaced by a nitrogen atom. In particular they found that the cyclic amidine derivative 2-aminopyridine had similar pressor properties. Fastier & MacDowall (1958a) then investigated the three isomeric aminopyridines. Despite the fact that only 2-AP is a true amidine, all three isomers had pressor activity. They also confirmed the analgesic properties of the aminopyridines as described by Von Haxthausen (1955a), but found that 4-AP was only 10% as effective as morphine, Fastier & MacDowall (1958b). In high doses all three isomers produced an increased excitability to touch and sound, tremor, clonic convulsions, tetany and death (Faster & MacDowall, 1958a).

Additional interest accrued to the aminopyridines with the discovery of their analeptic action by Shaw & Bentley (1949, 1952, 1955). Dogs narcotized to the point of unconsciousness with morphine, could be returned to a state of alert and intelligent wakefulness within two minutes after intravenous injection of derivatives of aminoacridine, aminoquinoline or aminopyridine. Shaw & Bentley (1952) reported that they were not able to arouse rats, cats or mice sedated with pentobarbitone, but Abernethy & Fastier (1958) found some degree of antagonism between 4-methyl - 2 aminopyridine and pentobarbitone. Yen, Sigg & Warner (1964) found that the effects of the tranquilizers diazepam and meprobamate could be antagonised by the pyridyl derivatives of benzoxidans and benz-dioxidans. In explaining their results they suggested that these agonists were not biologically active themselves, but were metabolized, and that one of the breakdown products was 4-AP which was responsible for the antagonism. In keeping with this suggestion they found that injections of

4-AP could antagonise these tranquilizing agents. High doses of the pyridyl derivatives produced violent and fatal convulsions as did the aminopyridines.

Potency of the different isomers

The toxicity of each isomer was quantified by Von Haxthausen (1955a), Fastier & MacDowall (1958a) and Yen et al (1964) by determining the dose which when injected intraperitoneally, produced a 50% mortality in a population of white mice, (ED₅₀). The results from each group is compiled and shown in Table 1.

	<u>Von Haxthausen (1955)</u>	<u>Fastier et al (1958)</u>	<u>Yen et al (1964)</u>
	mg/kg	mg/kg	mg/kg
2-AP	70	35	29.5
3-AP	30	28	19.5
4-AP	5	9	9.5

TABLE 1

They all found 4-AP to be the most potent. 3-AP was mostly found to be the next effective and 2-AP to be least effective.

Von Haxthausen (1955a) and Fastier (1958a) also determined the effectiveness of each isomer in raising the blood pressure of anaesthetised cats. Both found that 4-AP was most effective although they disagree about the relative potency of 2 and 3-AP. Fastier and MacDowall (1958a) also found that 4-AP was most effective in causing vasoconstriction in perfused rat hindquarters and increasing contractions of the rat phrenic nerve diaphragm preparation to direct and indirect stimulation.

By the early 1960's several independent investigations had thus focused attention on the pharmacological actions of the aminopyridines and shown 4-AP to be the most potent isomer. Since then interest has centred mainly on 4-AP and on its mode of action.

Action of 4-AP on synaptic transmission

That 4-AP could antagonise the paralizing actions of tri-iodoacetic acid, d-tubocurarine or neomycine, was reported by Lechat & Lemeignan (1967). This de-curarizing action could not be attributed to an anticholinesterase like activity of 4-AP since none could be demonstrated in vitro in rat serum (Lechat & Lemeignan, 1967). This antagonism of curare was confirmed on the isolated neuromuscular junction of the frog by Lemeignan & Lechat (1967) and on the phrenic nerve diaphragm preparation of the rat by Sobek, Lemeignan, Streighberger, Benoist, Gogovel & Lechat (1968). In an analysis of the membrane events at the frog neuromuscular junction Lemeignan & Lechat (1967) found that 4-AP caused an increase in the amplitude and duration of the miniature end-plate potentials and increase the amplitude of the end-plate potential; they came to the conclusion that the actions of 4-AP were both pre-synaptic and post-synaptic.

In experiments on cat motoneurones Lemeignan, Chanelet & Saade (1969) and Chanelet & Lemeignan (1969) studied the effects of intra-arterial injection or local micro-injection of 4-AP. The motoneurones became hyper-excitable, firing repetitively in response to a single Ia volley and also showed spontaneous activity. Spontaneous discharges were also recorded in the dorsal roots and were attributed to antidromic activity in primary afferent fibres.

These results suggested that 4-AP had not only effects at central synapses but also had a stimulating action on the nerve membrane. On frogs, Saade, Chanelet & Lonchamp (1971 a & b) found that local micro injection of 4-AP into the spinal cord produced a potentiation of reflexes supporting an action of 4-AP on the synapse.

Action on Nerve Cells

When 4-AP was applied to the isolated peripheral nerves of frog or cat (Lemeignan, 1970), the duration of the compound action potential was

increased to between 4 and 10 times normal. Using intracellular micro-electrodes Pelhate, Hue & Chanelet (1972, 1973a) examined the action of 4-AP on the giant axon of the cockroach and found the action potential to be prolonged and the negative after potential abolished. Axons also showed repetitive activity in response to a single stimulus. Pelhate et al (1973a) simply concluded that 4-AP "acted like TEA or DDT or Veratrine to modify the membrane conductance".

In further investigations on cockroach nerve, using constant current conditions Pelhate, Hue & Chanelet (1973b, 1974) and Pelhate, Hue, Pichon & Chanelet (1974) confirmed that 4-AP slowed the repolarizing phase of the action potential and in addition found that it did not alter the resting potential or the resting membrane conductance, neither did it affect the rate of rise of the action potential or its overshoot. This suggested that 4-AP acted to reduce selectively the delayed potassium conductance. Support for this suggestion came from voltage clamp experiments on cockroach nerve (Pelhate, Hue, Pichon & Chanelet, 1974) which showed that the early sodium current was not affected by 4-AP whilst the late potassium current was reduced, and could be abolished.

A communication on the 'Selective inhibition of the potassium current in the giant axon of the cockroach' was given to the Physiological Society in July, 1974 by Pelhate and Pichon. They reported 4-AP to be a very efficient and selective blocking agent of the potassium current in cockroach axons, about two thousand times more potent than TEA. It was this paper that was the starting point of this thesis.

In skeletal muscle, the potassium conductance is relatively insensitive to tetraethylammonium ions (Stanfield, 1970). Consequently, it is difficult to abolish this current in muscle when investigating other membrane events. For this reason the actions of 4-AP were examined on the potassium conductance of skeletal muscle in the hope that it would also be highly effective in this tissue. It was also hoped that by understanding the mode of action of 4-AP

in muscle this would reveal some of the structural details within that channel.

Physical Chemistry of the Aminopyridines

4-aminopyridine is an N-Hetero Aromatic Amine of general formula $C_5H_6N_2$. The molecule has two tautomeric forms, the amino and the dihydroimino form, as shown in Fig. 2.

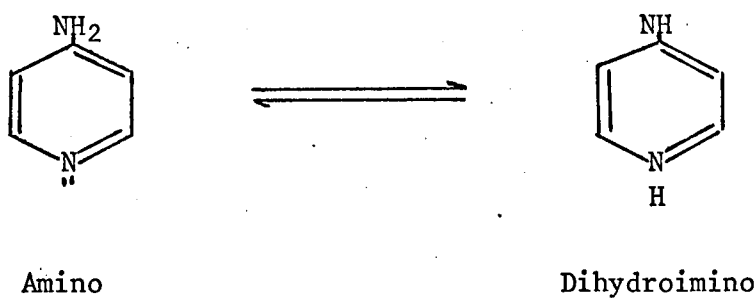


FIG. 2.

The tautomeric ratio is $2 \times 10^3 : 1$ in favour of the amino form (Angyal & Angyal, 1952; Mataga & Mataga, 1959). In aqueous solution 4-AP is a weak base and protonation normally occurs at the nitrogen atom within the ring (Albert, Goldacre & Phillips, 1948; Fastier & MacDowall, 1958a) as shown in Fig. 3.

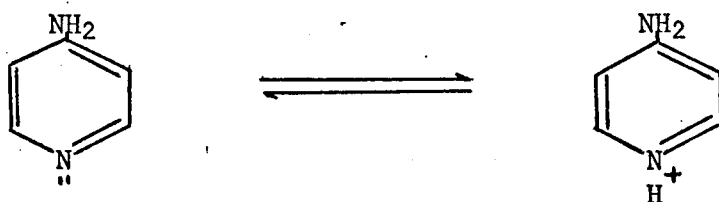


FIG. 3.

4-aminopyridine has a pK_a of 9.17 (Albert, Goldacre & Phillips, 1948); consequently it will exist at neutral pH in both the ionized and unionized form. At physiological pH, approximately 7.2, 99% of the 4-AP will be ionized and 1% unionized.

The 4-AP molecule has overall dimensions $4 \text{ \AA} \times 6 \text{ \AA}$ and is as thick as the Benzene residue (Christen, Morbury, Lister & Palmieri, 1975).

4-AP is one of three isomeric aminopyridines, the structures of which are shown in Fig. 4.

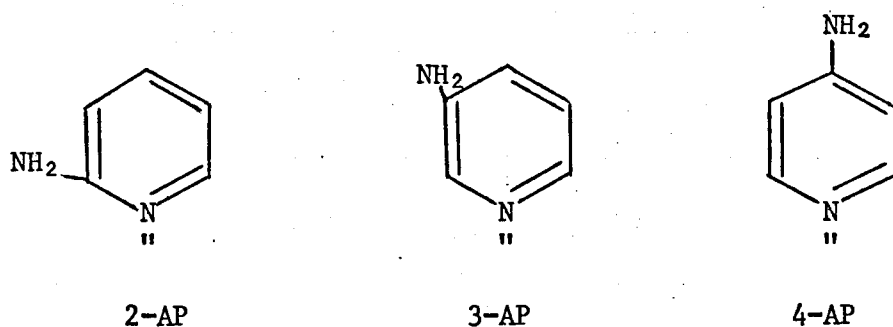


FIG. 4.

The isomers differ in their basic strength, pKa values for 4-AP, 3-AP and 2-AP are 9.17, 6.1 & 6.9 respectively (Albert, Goldacre & Phillips, 1948).

If the biological activity of 4-AP is the result of either the ionized or nonionized form it may be important to consider the following points. First, the cell surface pH may be as much as two pH units below the pH of the bulk solution (Danielli & Davies, 1951; Weiss, 1963); consequently, the degree of ionization of the weak base may be greater than anticipated. Secondly, the pKa value of such hetero aromatic bases may change if they are situated in close proximity to charged groups in or near the membrane. If a cyclic base (like 4-AP) were in close apposition to carboxyl ions or other δ -negative moieties, this would push electrons from the ring towards the basic site, thereby tending to increase the pKa (see Clark & Perrin, 1964).

Taken together these two conditions may lead to a greater degree of ionization of a base associated with a membrane than anticipated from the pH of the bulk extracellular solution.

The distribution and active form of a weak base

When a weak base such as 4-AP is added to the extracellular solution both the ionic and non-ionic forms will tend to diffuse across the cell membrane, but the hydrophobic non-ionic form is generally much more permeant. Once inside the cell, the non-ionic base may re-ionize if the intracellular pH is sufficiently low. Supposing that the internal pH does not alter materially as a result and that the permeability of the ionized form is negligible, then the system will come into equilibrium when the concentration of non-ionized base is the same on both sides of the membrane. The concentration of the ionized and unionized classes can then be calculated by application of the Henderson-Hasselbach equations to each compartment, thus:

$$\log \left[\text{BH}^+_{\text{o}} \right] - \log \left[\text{B}_{\text{o}} \right] = \text{pKa} - \text{pH}_{\text{o}} \quad 1.1$$

$$\log \left[\text{BH}^+_{\text{i}} \right] - \log \left[\text{B}_{\text{i}} \right] = \text{pKa} - \text{pH}_{\text{i}} \quad 1.2$$

where $\left[\text{BH}^+_{\text{o}} \right]$ and $\left[\text{BH}^+_{\text{i}} \right]$ are the concentrations of the ionized form outside and inside respectively, and $\left[\text{B}_{\text{o}} \right]$ and $\left[\text{B}_{\text{i}} \right]$ the concentrations of the non-ionized form outside and inside.

At equilibrium:

$$\left[\text{B}_{\text{o}} \right] = \left[\text{B}_{\text{i}} \right] \quad 1.3$$

and combining equations 1, 2 and 3 then yields

$$\log \frac{\left[\text{BH}^+_{\text{i}} \right]}{\left[\text{BH}^+_{\text{o}} \right]} = \text{pH}_{\text{o}} - \text{pH}_{\text{i}} \quad 1.4$$

For the specific case when the extracellular and intracellular pH are equal, then the concentration of the ionized form in each component will be the same:

$$\log \frac{\left[\text{BH}^+_{\text{i}} \right]}{\left[\text{BH}^+_{\text{o}} \right]} = 0 \quad 1.5$$

$$\Rightarrow \left[\text{BH}^+_{\text{i}} \right] = \left[\text{BH}^+_{\text{o}} \right] \quad 1.6$$

Direct measurement of the intracellular pH using hydrogen sensitive microelectrodes (Kostyuk, Sorokina & Kholodova, 1969) gives values for pH_{i} of 7.15 in frog muscle fibres, 7.34 in rat striated muscle and 7.26 in snail neurones; consequently the intracellular pH has a value close to the extracellular pH and the above mentioned conditions will apply.

By contrast, it can be seen from equation 1.4 that when the extracellular pH is made alkaline, then the concentration of ionized base within the cell will be higher than at pH 7.2 and vice versa if the extracellular pH is made acid with respect to the intracellular pH.

Table 2 shows how 1 mmole/l of a weak base with a pK_{a} of 9.17 (e.g. 4-AP) would eventually be distributed when applied to the external solution at different pH; the internal pH remaining constant at 7.17.

When the external pH is the same as the internal pH the intracellular and extracellular concentrations of ionized base will be equal at 0.99 mM. In acid solution, at pH 5.17, the extracellular concentration of non-ionized base is low, equation 1.1; as a result the intracellular concentration of ionized base is similarly low. If the external pH is made alkaline to pH 10.17, the concentration of non-ionized base increases and a considerable concentration of the ionized form can accumulate inside the cell. Under such conditions it is more than likely that the buffering capacity of the intracellular solution will become consumed with a consequent rise in intracellular pH. This will have a braking effect on the accumulation of the ionized form inside the cell: the high intracellular concentration calculated in alkaline solution would therefore not be reached.

It is possible to estimate by how much the intracellular pH may change as the concentration of weak base inside the cell is increased by assuming

TABLE 2

Distribution of 1 mmole/l of base (pKa 9.17) when applied in extracellular solutions of different pH.

	pHo = 7.17 (mM)	pHo = 5.17 (mM)	pHo = 10.17 (mM)
$[BH^+]_o$	0.990	0.9999	0.10
$[B]_o$ & $[B]_i$	0.001	0.00001	0.90
$[BH^+]_i$	0.990	0.001	900
pH _i assumed constant at 7.17.			

a value for the internal buffering capacity of muscle. From the law of Mass Action

$$\frac{[B_i][H^+_i]}{[BH^+_i]} = K_a \quad 1.7$$

where $[B_i]$ is the concentration of the unionized form and $[BH^+_i]$ the ionized form of a weak base; $[H^+]$ is the hydrogen ion concentration. Re-arranging equation (1.7) gives

$$\frac{[BH^+_i]}{[B_i]} = \frac{[H^+_i]}{K_a} \quad 1.8$$

$$\Rightarrow \frac{[BH^+_i]}{[B_i]} = 10^{(pK_a - pH_i)} \quad 1.8'$$

From the definition of Buffering capacity, then

$$pH_i = 7 + \frac{[BH^+_i]}{[N]} \quad 1.9$$

where $[N]$ is the internal buffering capacity in Slykes.

Combining equations 1.8' and 1.9 then the effect of $[B_i]$ on pH_i is given by:-

$$pH_i = \frac{7 + [B_i] 10^{(pK_a - pH_i)}}{[N]} \quad 1.10$$

$$\Rightarrow \frac{[B_i]}{[N]} = \frac{pH_i - 7}{10^{(pK_a - pH_i)}} \quad 1.10'$$

Fig. 5 shows how pH_i will vary with different concentrations of B_i for the arbitrary values of the internal buffering capacity; 10, 40 and 70 Slykes. (A figure of 30 Slykes has been reported by Furusawa & Kerridge (1927) for skeletal muscle). As the concentration of free base inside the cell is raised to approximately 0.01 mM the intracellular pH remains constant. With higher concentrations the intracellular pH begins to rise, gradually at first and then more steeply as the internal buffering capacity is consumed. Consequently, 1 mmole/l 4-AP applied externally at pH 5.0 and 7.2 will change the intracellular pH by less than 0.1 pH unit. In alkaline solution pH_i may rise by as much as 1 pH unit; in this case the concentration of ionized base inside would only rise to 90 mM.

The active class of a weak base

The question has often been asked as to which class of a base, i.e. the non-ionized molecules on the outside and inside, the ionized molecules on the outside or the ionized molecules on the inside, is responsible for its biological activity. One approach to this question has been suggested by Krah1, Keltch & Clowes (1940) and developed by Ariens & Simonis (1963); Ritchie & Ritchie (1968); Ritchie & Greengard (1961) and Narahashi, Frazier & Yamada (1970). The basis of this approach is the calculation of how the total extracellular concentration of base need be altered when the extracellular pH is changed in order to keep constant the concentration of one of the possible active classes. If it is assumed that the same concentration of the active class has the same action on the membrane, then at different external pH, different total extracellular concentrations of base will have the same effect, and for each possible active class the relationship between the iso-potent extracellular concentration and pH will differ in a characteristic way. Then by comparing the form of these calculated relationships with the experimental form the active class may be assessed.

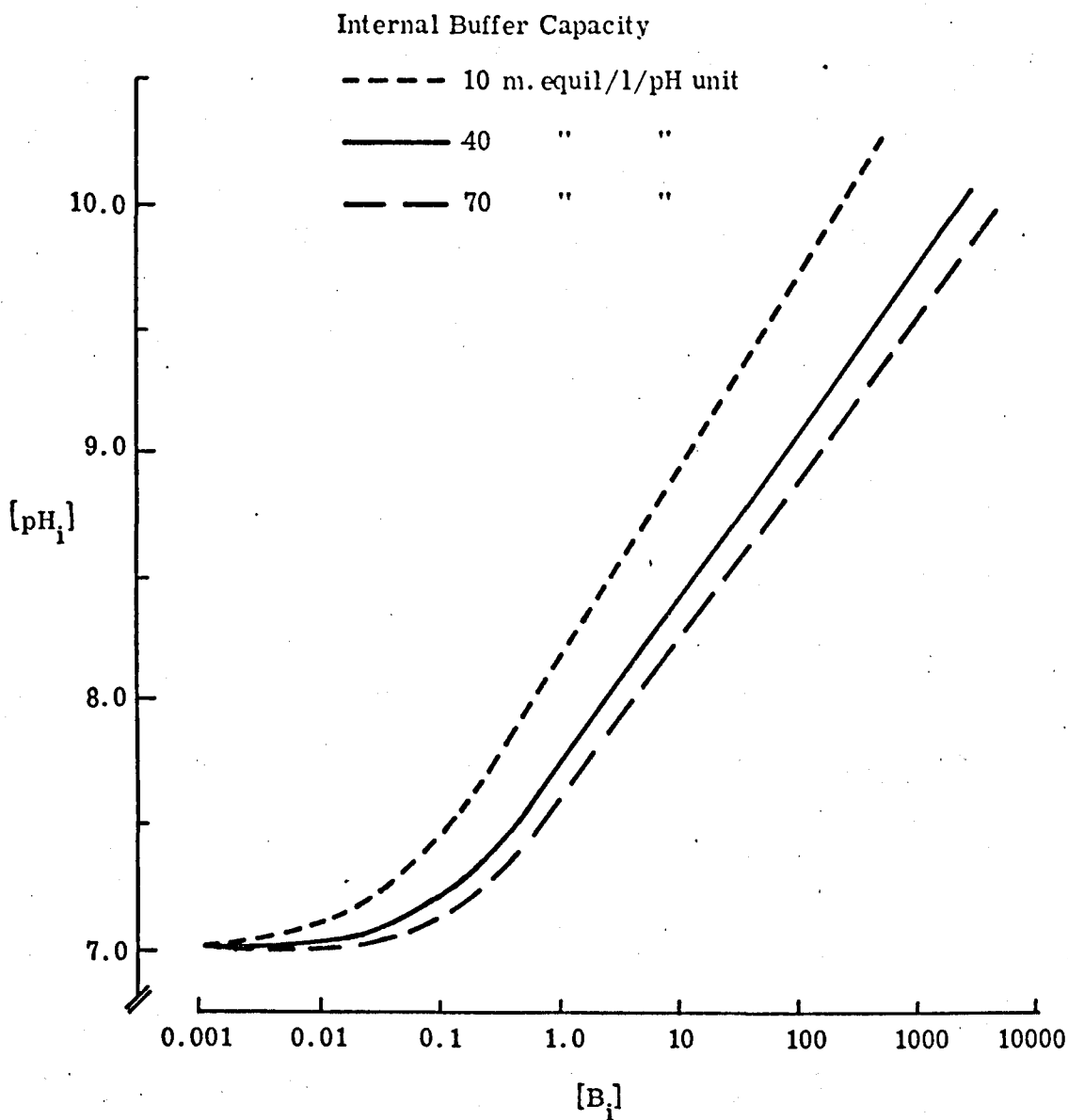


Fig. 5. The effect of non ionized free base inside a cell on the intracellular pH. Curves are drawn according to equation I_{10} for three assumed internal buffering capacities.

Experimentally, the potency is usually expressed as the total extracellular concentration required to produce half the maximal effect (ED_{50}). Fig. 6 shows the calculated relation for each possible active class of a weak base with pK_a 9.17. It is assumed that unit concentration of each active class is required to produce half maximal effect, and that the intracellular pH remains constant despite changes in the extracellular pH and the entry of base into the cell.

It may be seen that if the active form is the external cation, $[BH^+_o]$, the ED_{50} dose is constant up to about 2 pH units below the pK_a , it then begins to rise and eventually does so in a log-linear manner. On the other hand, if the effective class is the charged form inside, $[BH^+_i]$, or the uncharged form, then on raising the pH, the ED_{50} dose falls in a log-linear manner until the pK_a is approached; at pH values substantially higher than the pK_a the ED_{50} dose is constant. Supposing that the uncharged form and $[BH^+_i]$ have the same affinity for the site of action the ED_{50} curve for $[BH^+_i]$ will lie below the $[B_o]$ or $[B_i]$ curve by an interval determined by the difference between the pK_a and internal pH.

This approach has been successfully used by Ritchie & Ritchie (1968) and by Narahashi, Frazier & Yamada (1970), to determine the active class of many of the local anaesthetics which are weak bases. External application of the tertiary derivatives of lidocaine 6211 (pK_a 6.3) and 6603 (pK_a 9.8) suggest that the charged form inside the cell is the active class.

A similar approach can be used to determine the active class of a weak acid; for example the barbiturates. These molecules will distribute themselves across the membrane in the same way as a weak base. In contrast to the local anaesthetics, Narahashi, Frazier, Deguchi, Cleaves & Ernau (1970) have found that the active class of the barbiturates is the non-ionized molecule.

Thus when examining the actions of any weak base or acid, the above approach may be used to determine which of the three possible classes is responsible for the biological effect; this approach has been adopted in this

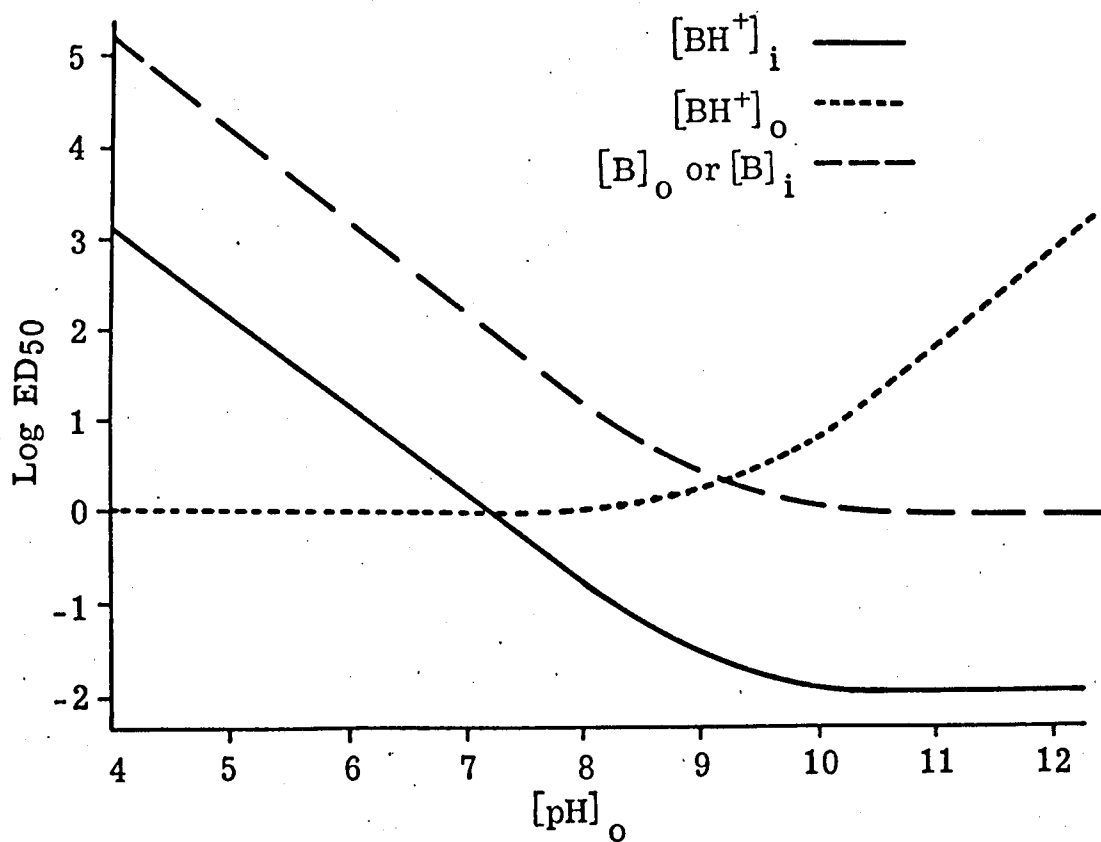


Fig. 6. Calculated ED₅₀—pH relationships for each possible active form of a weak base (pK_a 9.17). It is assumed that the internal pH remains constant at 7.17 and that only the non ionized base is freely permeable across the membrane and the effective concentration of each active form is unity.

thesis and applied to the actions of 4-AP in skeletal muscle.

I.iii The Potassium Conductance of Skeletal Muscle

The existence of a delayed increase in the potassium conductance upon depolarization in the skeletal muscle membrane has for long been supposed on grounds of indirect evidence. Fatt & Katz (1951) showed that the membrane conductance undergoes an increase which outlasts the duration of the action potential as in nerve. Hodgkin & Horowitz (1959) found that the amount of radioactivity labelled potassium that was lost during an action potential was 20 times greater than that anticipated from the resting efflux. In addition, evidence for an increase in potassium conductance upon depolarization by use of a constant current pulse was produced for fast skeletal muscle by Jenerick (1953) and slow skeletal muscle by Burke & Ginsborg (1956).

That skeletal muscle also contained an inwardly rectifying component of the potassium conductance was shown by Katz (1948) in muscles bathed in isotonic potassium solution. The presence of such a component in normally polarized fibres also emerged when the shunting effect of chloride ions was removed (Hutter & Noble, 1960).

The potassium conductance in muscle permanently depolarized with high K solution, is approximately that expected from the constant field theory, supposing no increase in potassium permeability. This and other findings (Nakajima, Iwasaki & Obata, 1962) led to the view that the increase in potassium conductance activated during the falling phase of the action potential undergoes inactivation upon prolonged depolarization.

Adrian & Freygang (1962) were the first to succeed in applying the voltage clamp technique to skeletal muscle. They space-clamped the pelvic end of skeletal muscle fibres bathed in isotonic K_2SO_4 solution, and characterized more fully the inwardly rectifying potassium conductance. Their analysis showed that beyond +50 mV the inwardly rectifying component ceases to make an appreciable contribution to the membrane conductance which is then dominated by the linear leak conductance. This linear component is

presumably present at all times and acts as a shunt. By the mid-1960's, therefore, it was recognized that the potassium conductance was composed of at least three components, a linear 'leak' component, the inward rectifier and the delayed rectifier.

The contractile apparatus of skeletal muscle becomes activated over a similar voltage range as do the ionic conductances. A major difficulty in the analysis of the conductance changes in skeletal muscle, therefore, is the mechanical events which normally attend depolarization; when several electrodes need to be impaled in a fibre, the risk of dislodgement and damage is especially high. Means for dissociating the electrical events from the mechanical events had therefore to be used before muscle fibres could be voltage clamped in the most interesting physiological range. One way in which this may be done, at least for short lasting depolarizations, is to treat the muscle with hypertonic solutions (Hodgkin & Horowitz 1957; Howarth 1958). By using this method and by working at low temperatures so as to slow the conductance changes and facilitate clamping through relatively high resistance electrodes, Adrian, Chandler & Hodgkin (1966, 1970a) succeeded in analyzing the conductance changes in muscle in a manner comparable to that employed on nerve by Hodgkin & Huxley (1952a).

As regards the delayed increase in potassium conductance, Adrian et al (1970a), found it to be activated by depolarizations beyond -55 mV. At -30 mV, the activated current rose along a sigmoid curve that reached a maximum in about 100 msec. Unlike nerve, however, the delayed potassium current in muscle is not maintained, and decays with an approximately exponential time course to zero. At 20°C the time constant for this decay was found to be 600 msec at -24 mV and 1000 msec at -40 mV.

The equilibrium potential for the delayed channel was determined in the usual way by repolarizing a fibre to different levels after turning on the potassium conductance. It was found that the reversal potential for the delayed current was between 10-15 mV positive to the resting potential.

This suggested that the delayed channel is not totally selective for potassium ions and that it may also conduct sodium ions. From the same experimental records Adrian et al (1970a), were also able to determine the instantaneous current voltage relation of the channel. In the voltage range -100 to 0 mV they found that the open channel behaved like an ohmic resistor. At 3°C the maximum conductance of the delayed rectifier varied between 8.5 mmho/cm² and 20 mmho/cm². The Q₁₀ of this relation was found to be 1.5.

The sigmoid time-course along which the currents activate was analyzed using the same procedure as Hodgkin & Huxley (1952d) on the potassium conductance of squid axons. As in nerve, the time-course of activation could be described by a first order reaction raised to the fourth power. The absolute values of the rate constants α_n and β_n in muscle were about half those in the squid axon; but in muscle they varied more steeply with potential. The rate constant of activation, $(T_n)^{-1}$, at 19°C was about five times that at 3°C, corresponding to a Q₁₀ of 2.4.

Adrian et al (1970a) also presented information on the degree of inactivation as a result of a depolarizing prepulse to different voltages. Using this classical two step experiment they obtained a Hodgkin-Huxley type steady state inactivation curve which could be described by the equation:

$$k_{\infty} = (1 + \exp(\frac{V + 40}{7.5}))^{-1} \quad 1.11$$

where k_{∞} is the fraction of delayed rectifier which can be activated after conditioning at voltage V. The implication, by analogy with the sodium system in nerve, is that potassium conductance in skeletal muscle is governed by two independent first order variables n and k; thus the time-course of the delayed increase in potassium conductance can be expressed:

$$g_K = \bar{g}_K n^4 k \quad 1.12$$

In addition to the three components of the potassium conductance already mentioned, Adrian et al (1970 a & b) described a fourth. Upon prolonged depolarization they found a slow component which reached a maximum in about 3 sec at -30 mV, and declined with a time constant of about 0.5 sec when the fibre was repolarized to -100 mV. This component also had a linear instantaneous current voltage relation, but the reversal potential of this channel was very near the resting potential, suggesting that it was more selective for potassium ions than the delayed channel.

Another approach to the study of the delayed potassium channel in skeletal muscle was used by Hutter (1970⁶⁹) and Argibay & Hutter (1973, 1974). They used muscles bathed in isotonic KCH_3SO_4 solution to which formaldehyde had been added. The use of isotonic KCH_3SO_4 solution ensured that only potassium ions carried the membrane current. Treatment with formaldehyde rendered the muscles non contractile and, conveniently also abolished the inwardly rectifying component of the potassium conductance (Hutter, 1970). A delayed outwardly rectifying conductance remains functional in the presence of formaldehyde. In fibres in which an artificial inside negative potential had been established by passing inward current, this conductance may be activated by a depolarizing step (Argibay, 1974). He showed that the time-course of the current could be described by equation 1.12; however, the rate constants of n , (i.e. α_n and β_n) were found to be 10 times slower under the above conditions than in fibres bathed in normal Ringer made hypertonic with sucrose.

The rate of inactivation, τ_k^{-1} , was found to be about 200 msec, i.e. greater than that in polarized preparations. This difference was not due to formaldehyde since detubulated fibres in isotonic potassium solutions also showed similar fast rates of inactivation. No variation in τ_k^{-1} with potential was found. The threshold of activation was found to be in the range -40 mV to -70 mV. The source of this variability is unknown. The maximum potassium conductance, \bar{g}_K , also varied considerably from fibre to

fibre and time to time. Argibay (1974) found \bar{g}_K to be 11 ± 3 mmho/cm².

When the development of inactivation was studied by testing the ability of a constant hyperpolarizing pulse to remove inactivation this process was found to follow a complex time-course which greatly outlasted the fall in current during long lasting depolarization. This led to the conclusion that the decay of the conductance reflects only the first overt step of a series of inactivating reactions, whose progress deepens the inactivation without electrical sign (Argibay & Hutter, 1973; Argibay, 1974).

It is interesting to note that in fibres bathed in isotonic KCH₃SO₄ Argibay (1974) did not find the slow component of the potassium conductance as reported by Adrian et al (1970). The reason for this discrepancy is at the present time unclear.

It is on such depolarized and formaldehyde treated preparations that the majority of the voltage clamp experiments in this thesis have been done. This preparation was chosen because it has several advantages over polarized preparations bathed in hypertonic ringer: (a) there is no mechanical response upon depolarization, (b) all the other components of the membrane ionic current are absent, (c) the other components of the potassium conductance, the inward rectifier and the slow component are absent. The formaldehyde treated preparation is therefore ideal for studying in isolation the delayed potassium channel in skeletal muscle.

II. METHODS

II. The three micro electrode voltage clamp technique

The three micro electrode voltage clamp technique used in this study is the same as that devised by Adrian, Chandler & Hodgkin (1966, 1970a). This technique was well established in the laboratory of Professor Hutter when I joined him and a detailed account of the theory and method prepared by a previous student in the laboratory (Argibay, 1974).

The aim of any voltage clamp technique is to uniformly polarize an area of excitable membrane and to determine the density of current which then flows across it. The relatively small size of skeletal muscle cells prevented the application of a similar voltage clamp technique to that developed for the giant axon of the squid by Cole & Curtis and Hodgkin & Huxley. However, a satisfactory technique, using three micro electrodes, was developed by Adrian, Chandler & Hodgkin (1966, 1970a) which took advantage of the special conditions which prevail at the end of a muscle fibre.

When a voltage is imposed near the end of a fibre it will decay towards the end according to the equation:

$$V_x = V_0 \cosh x/\lambda \quad 11.1$$

where V_x is the voltage at any distance, x , from the applied voltage which is V_0 ; and λ is the membrane space constant (Adrian et al, 1970). This relationship is in contrast to that which describes the decay of potential in the middle of an infinite cable; which is

$$V_x = V_0 \exp^{-x/\lambda} \quad 11.2$$

If λ is constant, then for a short distance from the voltage source, the hyperbolic function is much less steep than the exponential function; consequently, a voltage imposed near the end of a fibre will cause uniform polarization of the membrane at the end.

This situation is the basis for the voltage clamp method on skeletal muscle. The experimental set up for imposing and recording the uniform polarization is shown overleaf.

A current passing electrode (E_3) is impaled near the end of a fibre, as shown in Fig. 7, two other electrodes (E_2 & E_1) are inserted to record the potential in the end region.

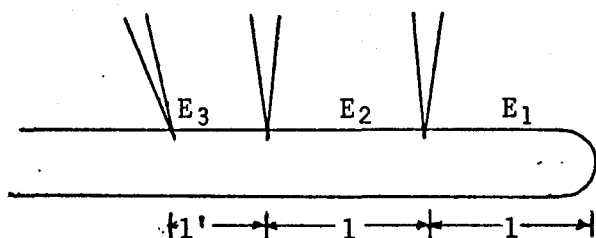


FIG. 7.

The electrodes are positioned at distances l , 1 and l' with respect to the end of the fibre. By injecting current through electrode E_3 a voltage imposed at E_3 will induce the same voltage at E_2 and only a slightly smaller voltage at E_1 . The difference between E_2 and E_1 can be calculated from equation 11.1, and is a consequence of current flowing across the membrane between electrodes E_2 and E_1 . This difference in potential between E_2 and E_1 can therefore be used as a measure of the current density at electrode E_1 (Adrian et al, 1970a).

In order to interpret the current density of any voltage it is necessary to have a uniformly polarized membrane such that all the voltage dependent events are in the same state. In muscle this is not possible since $V_2 > V_1$; hence the three micro electrode method can only approximate to the current density. The accuracy of the method depends on keeping the magnitude V_1 very close to V_2 . On the other hand if the difference is very small then there is difficulty in measuring $(V_2 - V_1)$ and consequently the current density. In practice a compromise is adopted and V_2 and V_1 are allowed to differ such that the error in determining the current density is less than 5% (Adrian et al, 1970a).

The difference between V_2 and V_1 may become large if the distance between the electrodes E_2 and E_1 is large compared to the membrane space constant, λ . The error in determining the current density is thus proportional to the ratio l/λ . It has been shown by Adrian et al (1970a) that if l/λ is kept below 0.65

then the error in estimating i_m will be less than 5%. In the present experiments the maximum membrane conductance is anticipated to be of the order of 30 mmho/cm²; this corresponds to a space constant of 430 μ m. An approximately uniform space clamp will then be possible under these conditions using an electrode spacing of no greater than 280 μ m.

$$i_m = \frac{2(V_2 - V_1)}{3\ell^2 r_i} \quad 11.3$$

where r_i is the internal resistivity of the fibre; a value of 180 Ω cm at 20°C is taken from Hodgkin & Nakajima (1972).

The membrane conductance per unit area can then be calculated using the expression

$$g_m = i_m/V_m = \frac{a(V_2 - V_1)}{3\ell^2 r_i V_1} \quad 11.4$$

where a is the radius of the fibre. If the radius, a , and the length, ℓ , are expressed in cm, and the internal resistivity in Ω cm, then equation 11.4 gives g_m in mmho/cm².

II. Apparatus

The voltage clamp apparatus used in these experiments is shown in Fig. 8. The membrane potential was recorded by electrode V_1 with reference to half cell (a) by means of a differential amplifier. In the early experiments a FENLOW FET AD 55 was used but in later experiments this was changed to a Clark Instruments WPI 750 differential amplifier. The output of this amplifier was then fed into the voltage clamp circuit. The first stage is a FENLOW A7 operational amplifier such that the overall gain of the two stages was 100. This signal was then compared in a SOLATRON AA 1023 operational amplifier with the command signal from the voltage programme unit. This unit provided up to four voltage steps of variable amplitude. The duration of each step was controlled in the early experiments using Digitimer 3290 and in the later experiments with a Digitimer D4030. The difference between the command signal and the amplified membrane potential was further amplified in a second SOLATRON amplifier which acted as a gain control. This stage was also fitted with capacity compensation in order to reduce oscillations. The final signal was then fed back to the preparation via the current electrode.

The voltage at V_2 was recorded at one side of a FENLOW FET AD 55 differential amplifier; the voltage at V_1 was fed into the other side, and the output, $(V_2 - V_1)$ recorded.

A TEKTRONICS 565 oscilloscope was used in all experiments. One channel was used to record V_1 and the other $(V_2 - V_1)$. The signals were photographed on 35 mm film (Kodak; RAR/2495) using a NIHON KODEN Automatic camera. The camera shutter was worked automatically using a gated pulse from a DIGITIMER 3290. In many experiments it was often necessary to record the beginning and the end of a long depolarizing pulse at a fast sweep speed. In this situation either the camera shutter was left open, and the oscilloscope made to sweep twice, thereby superimposing the beginning and end of the same frame: or alternatively, two separate photographs were taken.

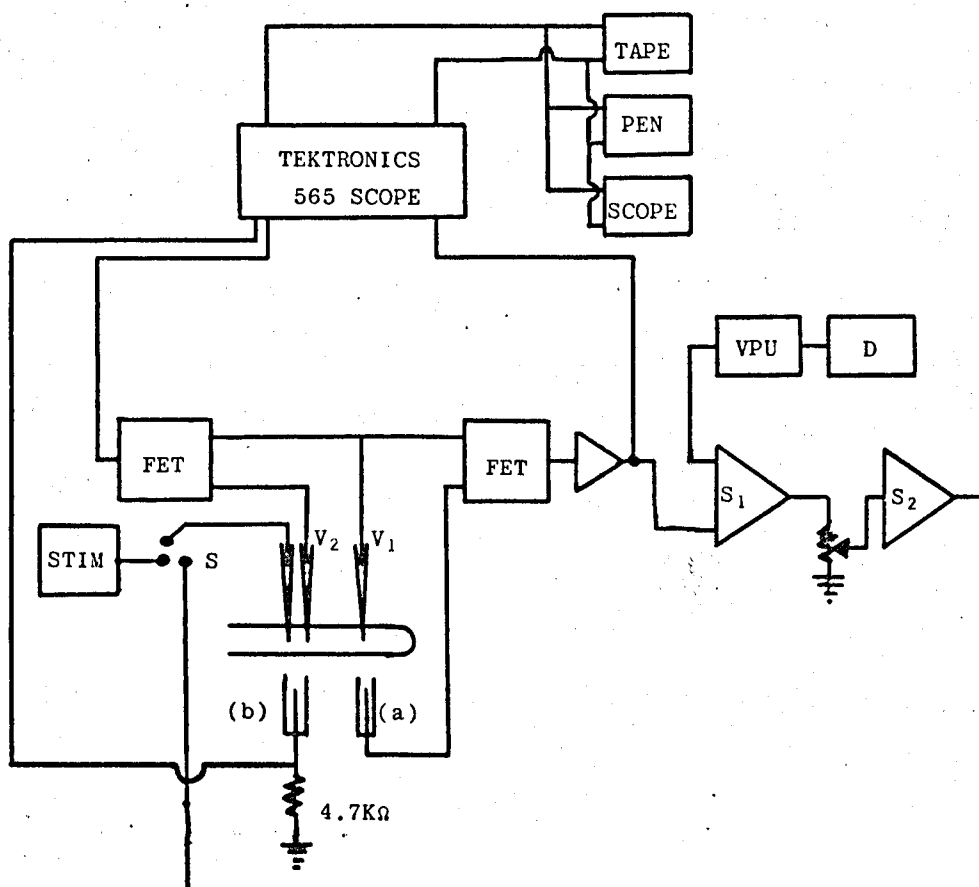


Fig. 8. Schematic diagram of the apparatus used. (a) and (b): Ag-AgCl half cells; D: digitimer; VPU: voltage programme unit; S: switch.

When a constant current situation was required switch, S, was closed, connecting a Devices 2533 isolated stimulator to the current electrode. In this situation the total electrode current was usually recorded as the voltage drop across a 4.7 K Ω resistor lying between the bath and earth potential and displayed on the oscilloscope.

Both channels of the Tektronics 565 oscilloscope were also fed into:

(a) A BRUSH 280 pen recorder, running at a low paper speed. This pen record served as a convenient record of the whole experiment and was used as a notebook to log solution changes, drug concentrations and other parameters of the experiment.

(b) Occasionally the signals were recorded on a BELL & HOWEL VR 3200 tape recorder. This facilitated the analysis of experiments as it permitted the reconstruction of the data at different gains and at different sweep speeds.

(c) In order to have immediate feedback on the experiment the signals were also displayed on a TEKTRONICS 5000 storage oscilloscope, which permitted the superimposing^{of} records.

II.iii Micro electrodes

Micro electrodes were of the conventional glass type. In early experiments electrodes were made from Corning Glass of wall thickness 0.012" and filled by boiling under vacuum. In later experiments fibre filled borosilicate glass from Clarke Electromedical Instruments was used: these electrodes were filled by capillarity by immersing in the appropriate electrolyte solution for 30 min to 1 hr before use.

Voltage recording electrodes were filled with 3 M KCl and only those electrodes with resistances between 15 to 40 M Ω and tip potential less than 5 mV were used. Where possible electrodes E₁ and E₂ were chosen such that they had similar tip potentials and resistance.

Current passing electrodes were filled with 1.8 M potassium citrate: electrodes between 5 and 10 M Ω were used. The rectifying characteristics of the current electrode was also tested and electrodes discarded if this was excessive.

II_{iv} Solutions

The composition of the basic experimental solutions is shown in table 3. To these basic solutions formaldehyde, 4-AP and sucrose were added as required.

Formaldehyde solutions were prepared by dissolving 0.9g of paraformaldehyde in 1500 ml of distilled water by continuous stirring at 70°C for 24 to 36 hours. The solution was then slowly cooled to room temperature and used to make the appropriate experimental solution. When the total volume of this stock solution is made up to 2000 ml the final concentration of formaldehyde is 15 mM.

Solutions that were to contain 4-AP (Sigma) were prepared immediately prior to use. 4-AP was added to the experimental solution as a small volume of aqueous solution and the pH of the experimental solution subsequently adjusted to the required value.

Sucrose (500 - 800 mM) was added to solutions when required.

Buffering of Solutions

Solutions were normally buffered with either TRIS/MALEATE/ACETYLGLYCINE or H_2PO_4 . In choosing the buffer for a particular solution two points had to be borne in mind. Firstly, phosphate containing solutions could not be made more alkaline than ~pH 7.8 since calcium phosphate is precipitated from solution. Secondly TRIS/MALEATE/ACETYLGLYCINE could not be used in conjunction with formaldehyde since formaldehyde reacts with TRIS to render TRIS and formaldehyde ineffective. A problem therefore arises when formaldehyde containing solutions at pH 9.8 are required. In the event no buffer was used; the pH of such solutions being carefully adjusted with KOH. If such a solution was kept in a covered reservoir, the pH remained reasonably stable for up to 1 hr. The pH of alkaline solutions containing 4-AP was much more stable due to the buffering capacity of 4-AP itself.

In all the voltage clamp experiments done at pH 5.0 and pH 7.2, Results sections i, ii, iii, vi, vii, viii, phosphate was therefore the buffer of choice. In section iv, on the effect of 4-AP on the action potential TRIS buffer was used. This was possible on account of the fact that formaldehyde could not be used. Formaldehyde by itself modifies the action potential. In these experiments the twitch which normally attends the action potential was abolished by making the Ringers solution, (Solution A), hypertonic with 500 mM sucrose.

In section v, the effect of 4-AP on the Inward Rectifier, isotonic KCH_3SO_4 solutions containing TRIS buffer were used. No formaldehyde was used in these experiments since it abolishes the inward rectifier; however the solution was not required to be hypertonic since the isotonic potassium solution depolarizes the fibre making it mechanically refractory.

SOLUTIONS

TABLE 3

SOLUTION	Na ⁺	K ⁺	R _b ⁺	Ca ²⁺	M _g ²⁺	Cl ⁻	CH ₃ SO ₄ ⁻	TRIS/MALEATE	ACETYL-GLYCINE	H ₂ PO ₄	GLYCEROL
A	112.5	2.5	-	1.8	-	118.3	-	2.0	2.0	-	-
B	112.5	2.5	-	1.8	-	1.8	114.5	2.0	2.0	-	-
C	-	114.5	-	1.8	-	1.8	114.5	2.0	2.0	-	-
D	-	114.5	-	1.8	-	1.8	114.5	-	-	0.85	-
E	-	114.5	10.0	1.8	-	11.8	114.5	-	-	0.85	-
F	-	114.5	-	1.8	-	1.8	114.5	-	-	-	-
G	112.5	2.5	-	1.8	-	111.8	-	2.0	2.0	-	-
H	112.5	2.5	-	5.0	5.0	125.0	-	2.0	2.0	-	500

II_v Muscle preparation

All experiments were done on sartorius muscle fibres of the frog (*Rana Temporaria*). The animals were stored in polythene boxes at a temperature of approximately 6°C. Both summer and winter frogs were used and no obvious difference was observed in the results between these groups. Frogs were killed by stunning and pithing. The sartorius muscle was then removed along with a slip of the pelvic girdle onto which the muscle is attached. The preparation was then pinned out on dental wax using fine entomological pins and all superficial connective tissue removed. With the ventral side of the muscle uppermost the pelvic end was carefully cleaned such that the point of insertion of all the fibres into the tendon could be seen.

From this point the procedure depended on the experiment to be done. If the experiment required, Cl ions were replaced by CH_3SO_4 ions by washing the muscle in solution B. When all fibrillation due to Cl withdrawal had ceased a further 10 min was allowed for complete Cl withdrawal.

If a depolarized preparation was then required the muscle was then subjected to isotonic potassium solution, solution C. First the muscle was unpinned at the distal end and held at a slack length. Isotonic KCH_3SO_4 was applied which resulted in a strong contracture; the muscle was not allowed to shorten beyond its slack length. Upon relaxation from the contracture the muscle was carefully returned to its resting length. The preparation was then transferred into a perspex bath of volume 0.8 ml and securely pinned down at the pelvic end; the muscle was then stretched to 1.2 times resting length.

The bath was then positioned in the recording set up and solution run into the bath at a rate of approximately 3 to 6 ml/min. Solution was constantly withdrawn from the bath by suction such that the level remained constant. A six way tap positioned adjacent to the inlet to the bath allowed switching of the perfusing solution with minimal disruption to the solution flow.

The majority of the experiments were done at room temperature (19 to 24°C). In some experiments the solutions were cooled to approximately 5°C prior to their addition to the bath. Under these circumstances the bath temperature and consequently the tissue temperature is reduced to approximately 8°C.

II. vi Detubulation Procedure

Isolated and cleaned sartorius muscles were pinned taut on a piece of dental wax and placed in a small beaker containing solution G, (500 mM glycerol). The muscle was kept in this solution at 20°C for between 90 and 120 min. After this period of glycerol loading the muscle was transferred to a second beaker containing solution H for detubulation. This solution was stirred constantly to increase the effectiveness of the osmotic shock. After approximately 60 min in solution H, the muscle was returned to solution A for approximately 30 min. At the end of this procedure most of the fibres had recovered their normal diameter. Muscles were then prepared by taking them through solution A - C as described. Detubulation was successful in the majority of fibres, but some still showed a mechanical response upon depolarization after a period of repriming; other fibres demonstrated abnormally high leak conductances; such fibres were rejected. Fibres which had a low leak conductance and gave no mechanical response to depolarization were used in these experiments.

III. RESULTS

III.

Early experiments on the actions of 4-aminopyridine on the delayed potassium currents.

Fig. 9 shows a family of delayed potassium currents recorded in a formaldehyde treated fibre in isotonic KCH_3SO_4 solution in the absence of 4-AP. Upon depolarization from the holding potential^{OF -122mV} the delayed current activates along a sigmoid curve, reaches a peak and then decays with an exponential time course to zero. Such records are similar to those obtained by Hutter with this preparation and shown by Argibay (1974). Fig. 10 shows the progressive action of 1 mmole /l 4-AP (pH 7.2) on such formaldehyde treated delayed potassium currents. Outward currents in response to a depolarizing step to +30 mV, and inward currents in response to a depolarizing step to -30 mV were recorded at the times indicated after the addition of 4-AP to the bathing solution. In the absence of 4-AP (records A and B) the delayed currents activate along a sigmoid curve, reach a peak and then inactivate with a single exponential time constant to zero. After the addition of 4-AP, the magnitude of the currents flowing in the inward and outward direction is progressively reduced and an initial faster component of inactivation develops at both potentials. These changes are complete after approximately 15 min exposure to 4-AP. At that time, the peak current determined at +30 mV and -30 mV is reduced in this case by 34% and 77% respectively. The time to peak of the inward current is reduced from 67 msec to 49 msec, and the time course of inactivation is no longer a single exponential decay. The decay now consists of an initial fast and later slow component. The fast component of induced inactivation is present at both membrane potentials but is more pronounced at the more positive potential.

A common feature in such experiments was a small increase in the peak conductance within the first minute of adding 4-AP to the bath. In the experiment shown in Fig. 10 the peak conductance at $t = 0$ is 28.0 mmho/cm^2 ,

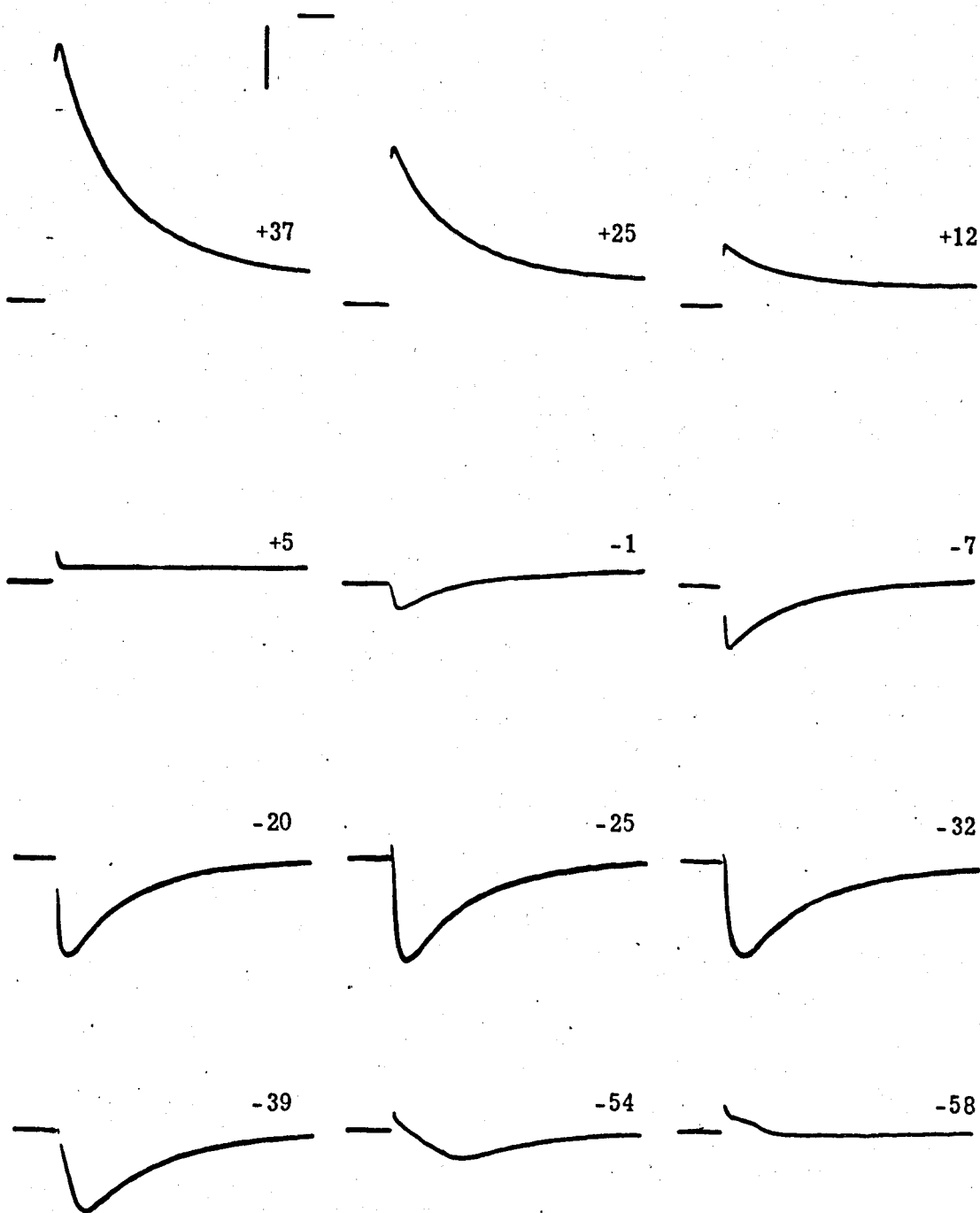


Fig. 9. Delayed potassium currents in a formaldehyde treated fibre in the absence of 4-AP. Current traces were recording during depolarizing steps to the potentials shown at the end of each trace (mV). Vertical calibration 0.21 mA/cm^2 ; horizontal calibration 20 msec. Fibre 92761; $l=200\mu$; $l'=50\mu$; $a=100\mu$; Temp. 20°C .

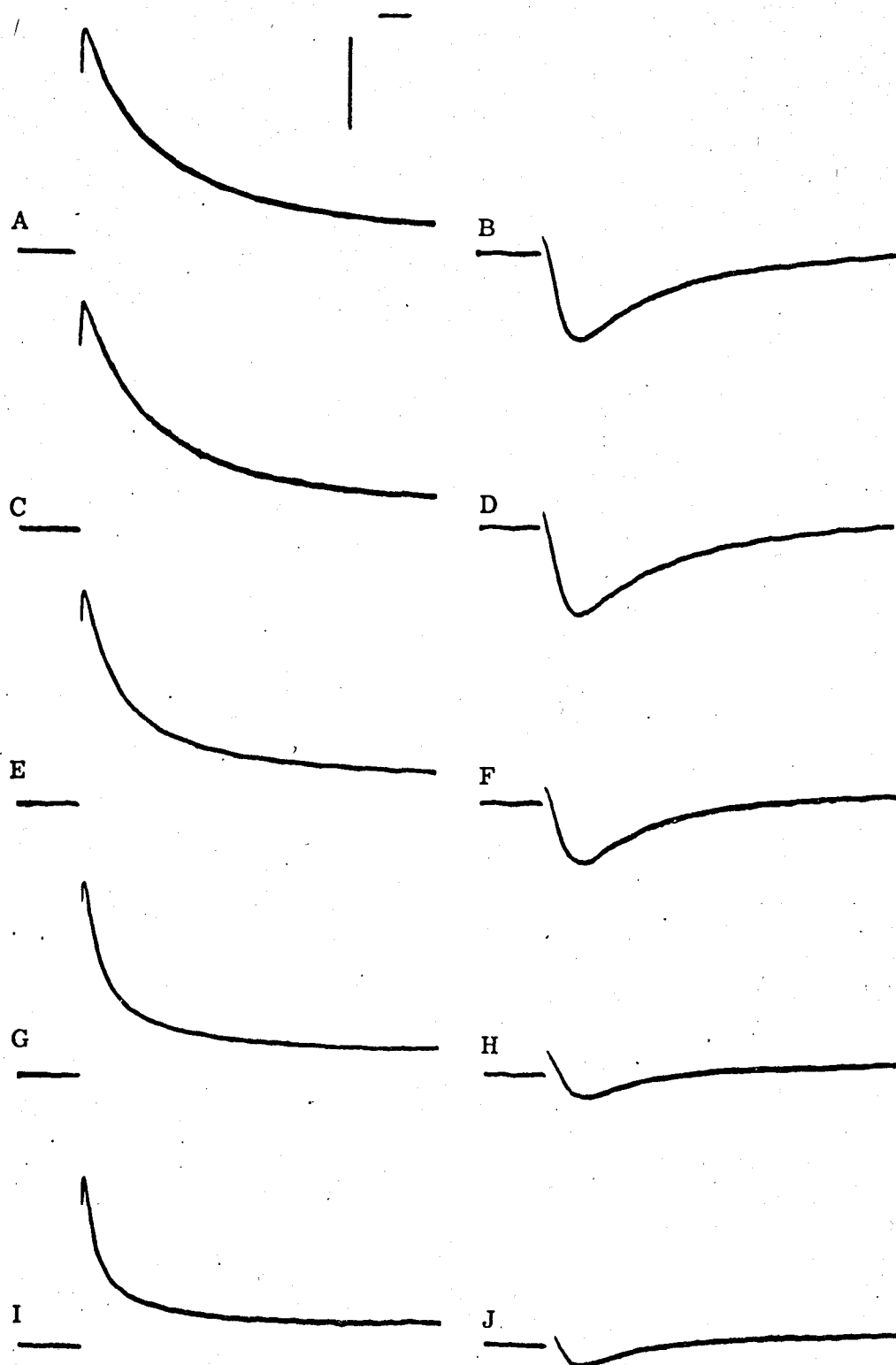


Fig. 10. The effect of 1 mmole/l 4-AP (pH 7.2) on delayed outward ($V_t = +30\text{mV}$) and inward ($V_t = -30\text{mV}$) potassium currents. Records A and B are control records in the absence of 4-AP; the remainder were recorded after the addition of 4-AP at times; C=30 sec; D=60 sec; E=6min; F=7min; G=9min; H=11min; I=14min; J=15min. Fibre F1 090276: $I = 200\mu$; $I' = 50\mu$; $a = 100\mu$; vertical calibration 0.29 mA/cm^2 ; horizontal calibration 20 msec; Temp. 21°C .

whereas at $t = 30$ sec the peak conductance is 29.5 mmho/cm . At this time the concentration of 4-AP in the bath is unknown, but is likely to be much less than 1 mM due to mixing with the 4-AP free solution initially in the bath: this increase suggests that at low concentrations 4-AP may have a stimulating effect on the delayed potassium channel.

Fig. 11 shows a family of delayed currents recorded in a different fibre in the presence of 1 mmole/l 4-AP (pH 7.2). In each record the time course of inactivation is no longer a single exponential decay and consists of an initial faster and later slow component. This initial faster component is present at all membrane potentials but more so for positive voltage steps.

The action of 4-AP on the potassium conductance of detubulated muscle fibres

The delayed potassium channel can also be studied in detubulated fibres bathed in isotonic KCH_3SO_4 (Argibay, 1974). These experiments have been done to determine the actions of 4-AP on fibres that are not treated with formaldehyde but which shows no mechanical response on depolarization.

Fig. 12 shows typical examples of the membrane current in a detubulated fibre bathed in isotonic KCH_3SO_4 solution. Upon depolarization from the holding potential the delayed potassium channel is activated and transient currents similar to those shown in Fig. 9 in the formaldehyde treated fibres can be recorded. Fig. 13 shows the progressive actions of 1 mmole/l 4-AP (pH 7.2) of the delayed outward ($V_t = +30 \text{ mV}$) and inward ($V_t = -30 \text{ mV}$), potassium current in a detubulated fibre. Upon exposure to 4-AP both the inward and outward delayed current is reduced by 4-AP. As with the fibres treated with formaldehyde the inward current is reduced to a greater extent than the outward current, 80% and 20% respectively; the time to peak is reduced and there is an additional and faster component of inactivation which is more pronounced at the more positive potential. There is also a small increase in peak conductance within the first minute of adding 4-AP

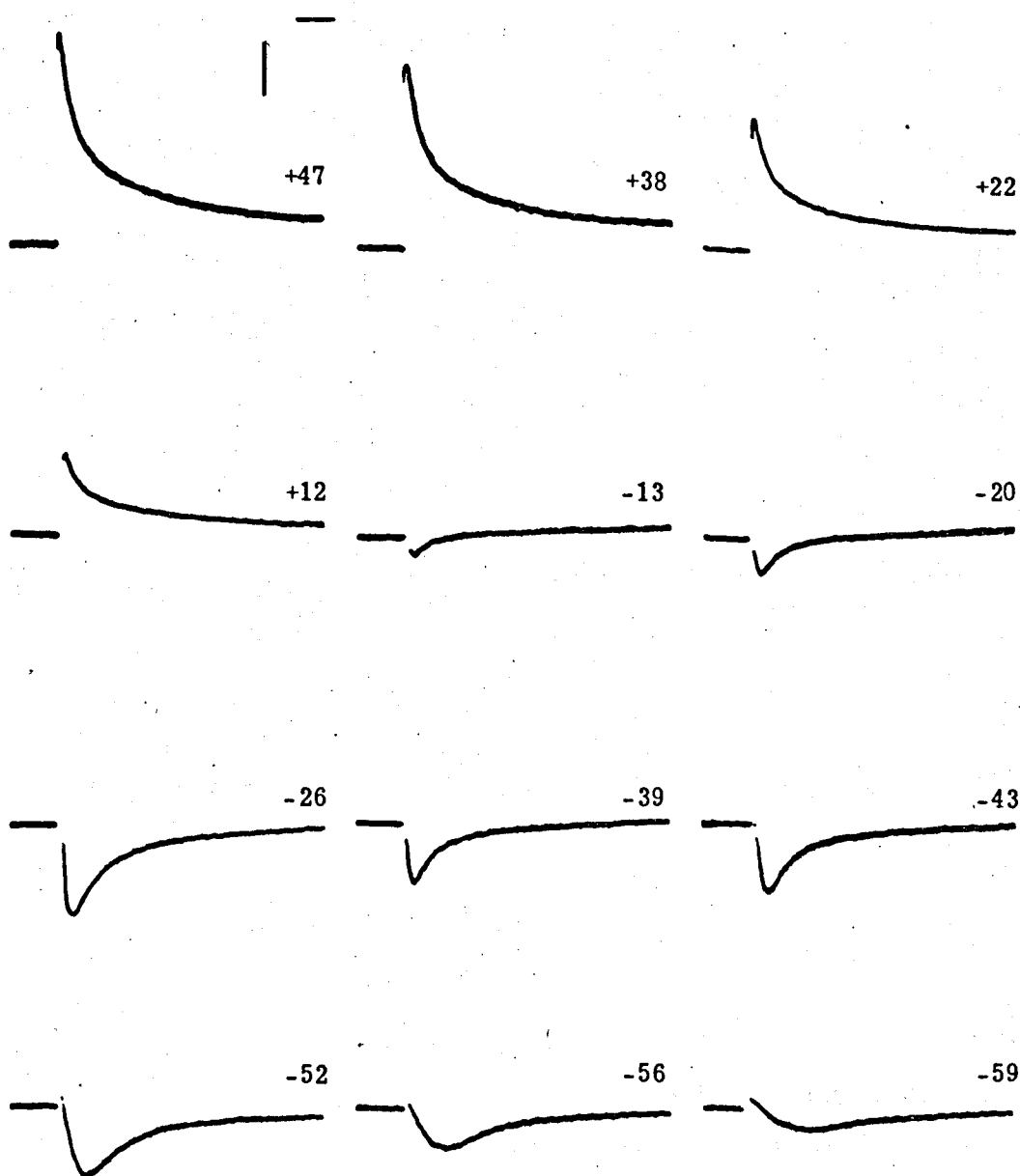


Fig. 11. Delayed potassium currents recorded in a formaldehyde treated fibre in the presence of 1 mmole/l 4-AP (pH 7.2). Current traces were recorded during depolarizing steps to the potentials shown at the end of each trace (mV). Vertical calibration 0.094 mA/cm²; horizontal calibration 20 msec. Fibre F1 10276: $l=150\mu$; $l'=50\mu$; $a=80\mu$; Temp. 20°C.

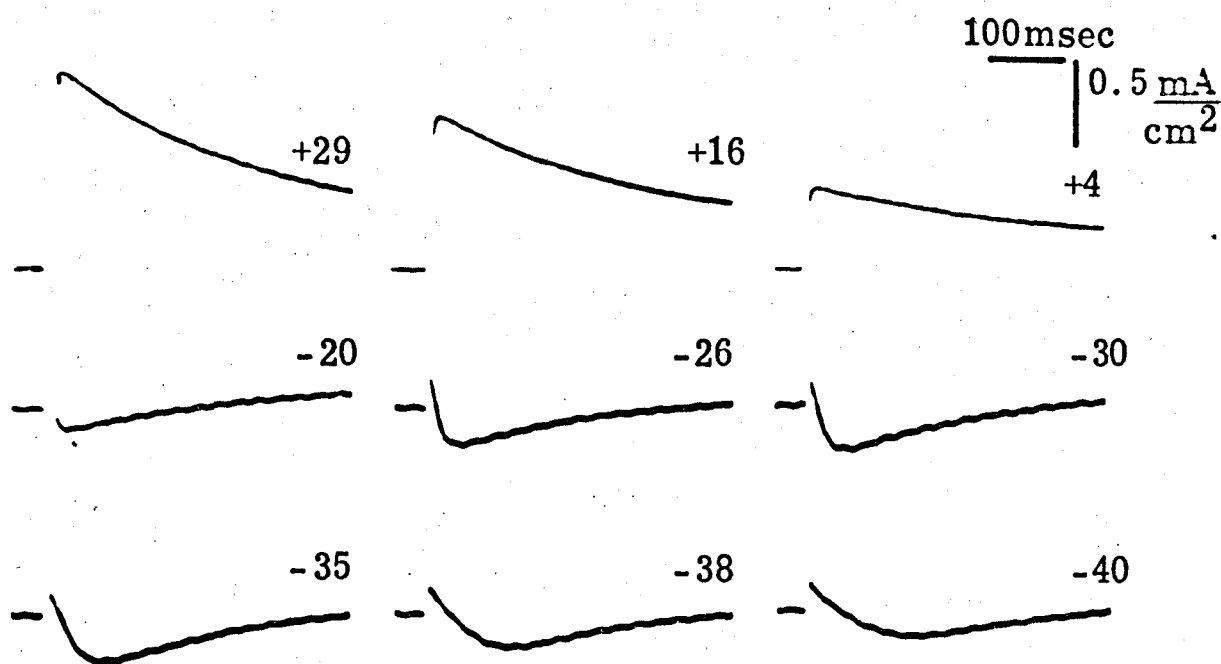


Fig. 12. Membrane currents recorded in a detubulated fibre bathed in isotonic KCH_3SO_4 in the absence of 4-AP. After repriming at -122 mV for 15 sec the membrane was depolarized to the level shown by the figure at the end of each trace (in mV). Fibre F1 01675, $l=200\mu$; $l'=50\mu$; $a=80\mu$; Temp. 20°C .

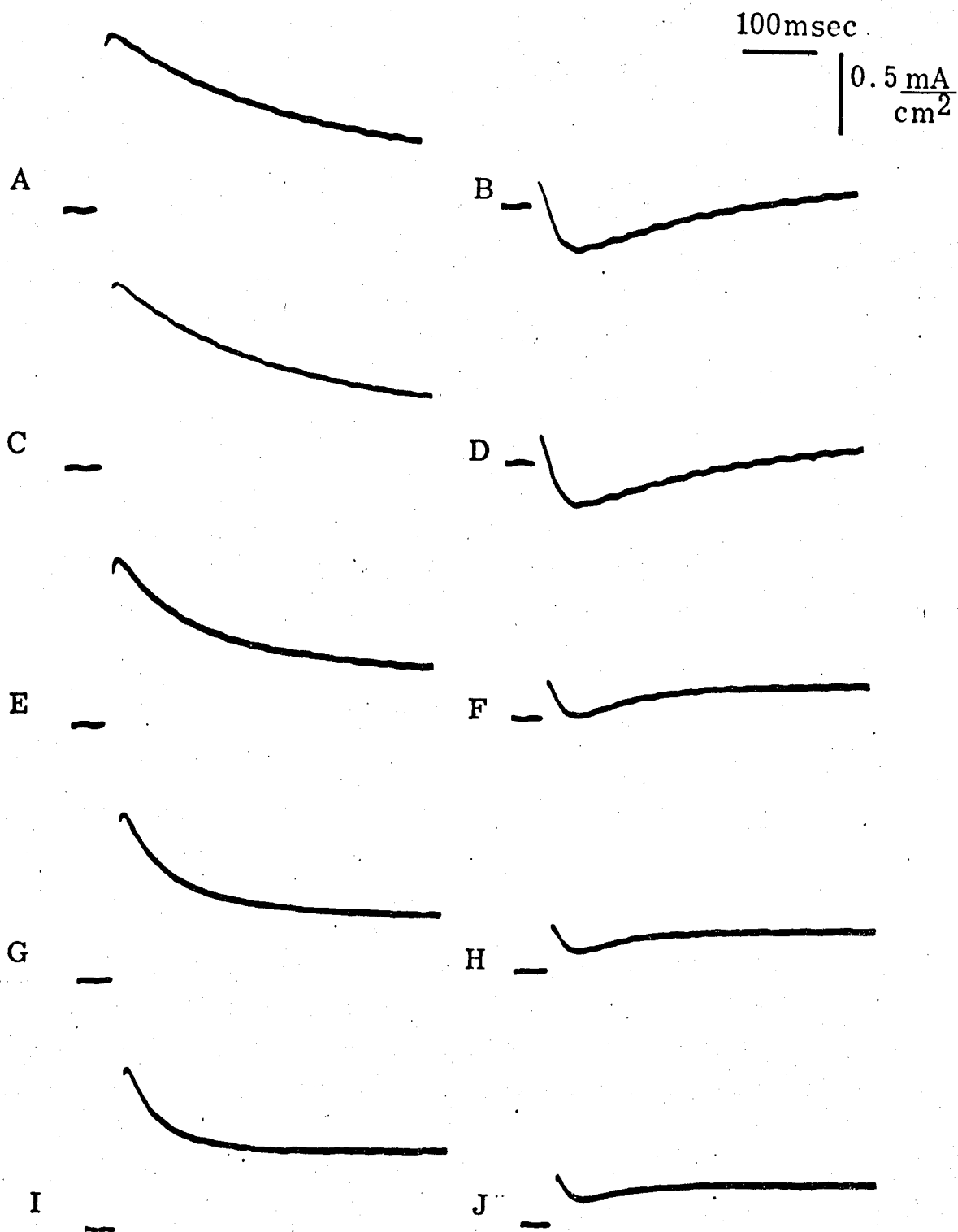


Fig. 13. The progressive actions of 1 mmole/l 4-AP (pH 7.2) on delayed outward and inward currents in a detubulated fibre. A and B are control records in the absence of 4-AP: remaining traces recorded at times C=2.0 min; D=3.0 min; E=3.5 min; F=5.0 min; G=6.0 min; I=7.5 min; J=8.0 min. Records A,C,E,G,I depolarization to +30 mV; records B,D,F,H,J depolarization to -30 mV. Same fibre as Fig. 12.

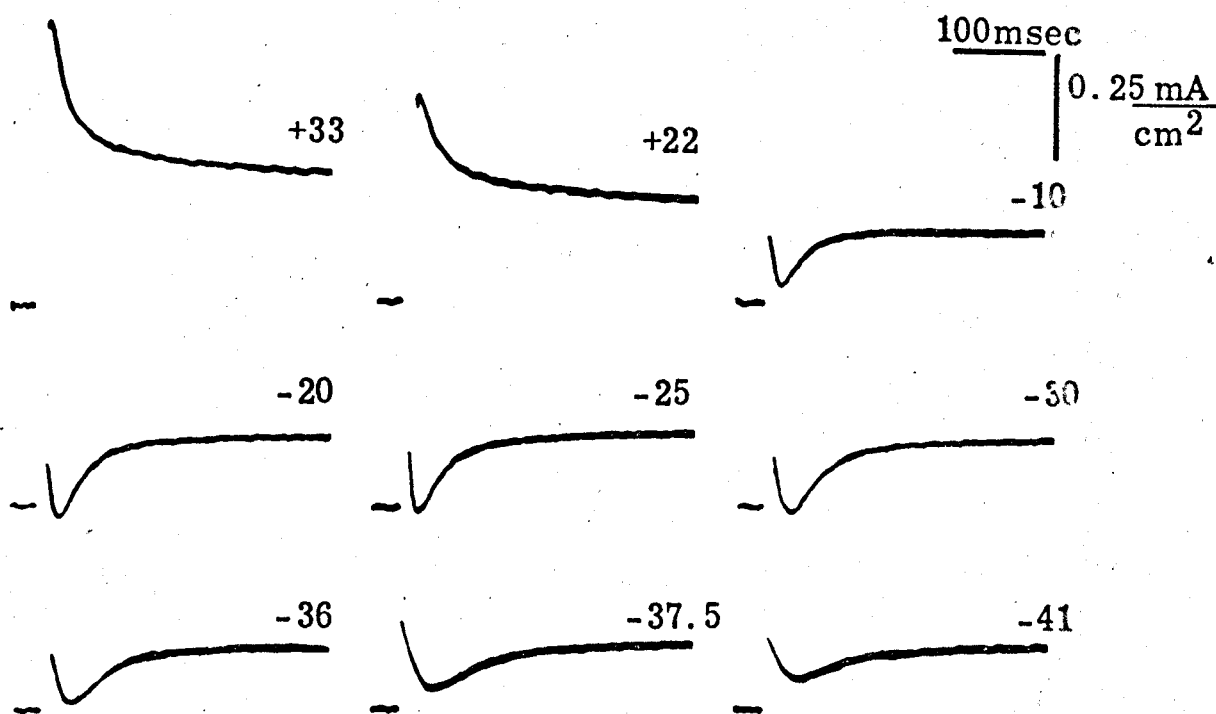


Fig. 14. Membrane currents in the presence of 1 mmole/l 4-AP (pH 7.2) in a detubulated fibre in isotonic KCH_3SO_4 . For each record the level of depolarization is shown on the right (in mV). Fibre F2 01675 $l=250\mu$; $l'=50\mu$; $a=70\mu$; Temp. 20°C .

to the bathing solution. At $t = 0$ the peak conductance is 32 mmho/cm^2 whilst after 1 min exposure the peak conductance is 34 mmho/cm^2 .

Fig. 14 shows records of the membrane current for another detubulated fibre exposed to 1 mmole/l 4-AP (pH 7.2). The magnitude of the depolarizing step is shown beside each record. The induced faster component of inactivation is present at all membrane potentials and occurs in the presence of outwardly and inwardly flowing current, and proceeds at a faster rate as the internal potential is made more positive. This family of current records which show the blocking action of 4-AP on detubulated fibres is very similar to that shown in Fig. 11.

These experiments confirm the earlier findings of Argibay (1974) for the continued functioning of the delayed potassium channel in formaldehyde treated fibres. They also show that the blocking actions of 4-AP are similar in detubulated and formaldehyde treated fibres.

The analysis of the blocking actions of 4-AP has been done on the more convenient formaldehyde treated fibres.

The effect of 4-AP on the peak potassium conductance

In many experiments it was possible to determine, in the same fibre, the peak membrane conductance at different voltages before and after treatment with 4-AP. The peak conductance was used, rather than \bar{g}_K because of uncertainties regarding the extrapolation of the decay processes in the presence of 4-AP to zero time.

Fig. 15 shows a semilogarithmic plot of the voltage dependence of the peak potassium conductance before (open symbols) and after (filled symbols) the addition of 1 mM 4-AP to the bathing solution. In the absence of 4-AP the peak conductance is strongly dependent on potential in the range -60 mV to -20 mV and undergoes an e-fold increase every 8 mV ; beyond $+10 \text{ mV}$ the peak conductance reaches a maximum which in this case is 28.6 mmho/cm^2 .

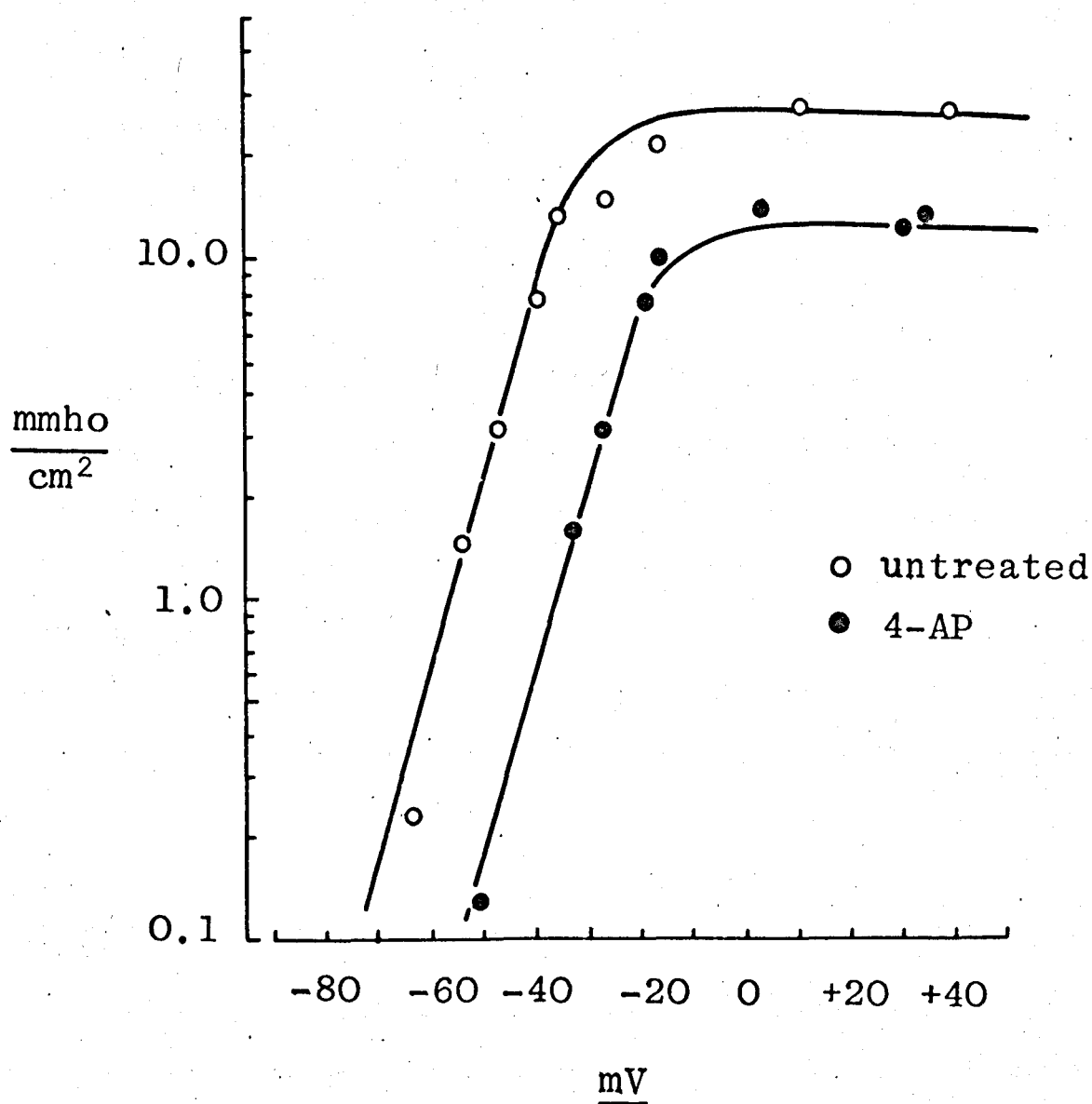


Fig. 15. The effect of 1 mmole/l 4-AP (pH 7.2) on the peak potassium conductance: in the absence (○) and after 15 min equilibration with 4-AP (●). Curves drawn according to equation III₁; constants shown in Table 4. Fibre 6575I $l=200\mu$; $l'=50\mu$; $a=80\mu$; Temp. 20°C .

In the presence of 4-AP the peak conductance is reduced at all membrane potentials. In the potential range -50 to -20 mV the peak conductance remains highly sensitive to potential and undergoes an e-fold increase every 8 mV. At positive potentials the peak conductance also reaches a maximum, and has a value of 13.6 mmho/cm² which is 48% of that in the untreated fibre.

In both the treated and non treated fibres the data can be fitted by an equation of the form:

$$(g_K)_V = \bar{g}_K (1 - \exp - \frac{V - \bar{V}}{k})^{-1} \quad 111.1$$

where $(g_K)_V$ is the conductance at any voltage V ; \bar{g}_K is the maximum available conductance; \bar{V} can be used as a measure of the threshold and k determines the voltage dependence of the relation in its most sensitive range.

Table 4 presents the results from eight experiments which have been analyzed in terms of equation 111.1. The mean reduction in the maximum potassium conductance is $48 \pm 6\%$ (\pm S.D.) and the mean shift in the threshold is 19.5 ± 9.8 mV (\pm S.D.). The value of k is not systematically altered in the presence of 4-AP.

The effect of 4-AP on the voltage dependence of steady state inactivation

The fraction of channels which undergo activation and inactivation depends on the membrane potential. At any membrane potential the fraction of channels which activate can be determined from a single depolarizing step and the fraction of channels which have inactivated at that potential can be determined by imposing a second strong depolarizing step and relating this to the maximum conductance. The effect of 4-AP was examined on the voltage dependence of the steady state inactivation. Fig. 17(a) shows the voltage protocol used in such an experiment; and Fig. 16 shows examples of the current records obtained in the same fibre before and after the addition of 4-AP. Fig. 17(b) shows a plot of the data in Fig. 16, before 4-AP, open

TABLE 4

The effect of 1 mmole/l 4-AP (pH 7.2) on the delayed rectifier

	(1)	(2)	(3)	(4)	(5)	(6)	(7)	(8)
	mmho/cm ²			mV				
FIBRE	$\bar{g}_{KNormal}$	\bar{g}_{K4-AP}	\bar{g}_{4-AP}/\bar{g}_N	\bar{V}_N	\bar{V}_{4-AP}	ΔV	k_N	k_{4-AP}
6.5.75 I	42.0	18.0	0.43	43.0	23.5	19.5	12.0	9.9
30.4.75 I	28.1	12.8	0.46	37.0	18.5	18.5	8.8	10.9
1.5.75 I	28.9	12.0	0.42	32.8	23.8	9.0	9.5	10.1
8.5.75 I	24.5	12.0	0.49	37.0	13.0	24.0	14.9	12.2
F1 18.2.76 I	27.5	12.8	0.47	35.0	21.8	14.8	7.1	6.8
F1 18.2.76 II	24.8	13.8	0.55	35.4	21.0	14.4	8.2	9.4
F2 26.2.76 I	26.7	15.0	0.56	34.5	20.0	14.5	6.5	8.2
25.10.75 I	<u>11.7</u>	<u>4.7</u>	<u>0.42</u>	63.0	22.0	41	4.1	8.4
Mean \pm S.D.	26.8 \pm 8.2	12.6 \pm 3.8	0.48 \pm 0.06	39.7 \pm 9.9	20.5 \pm 3.5	19.5 \pm 9.8	8.9 \pm 3.3	9.5 \pm 1.1

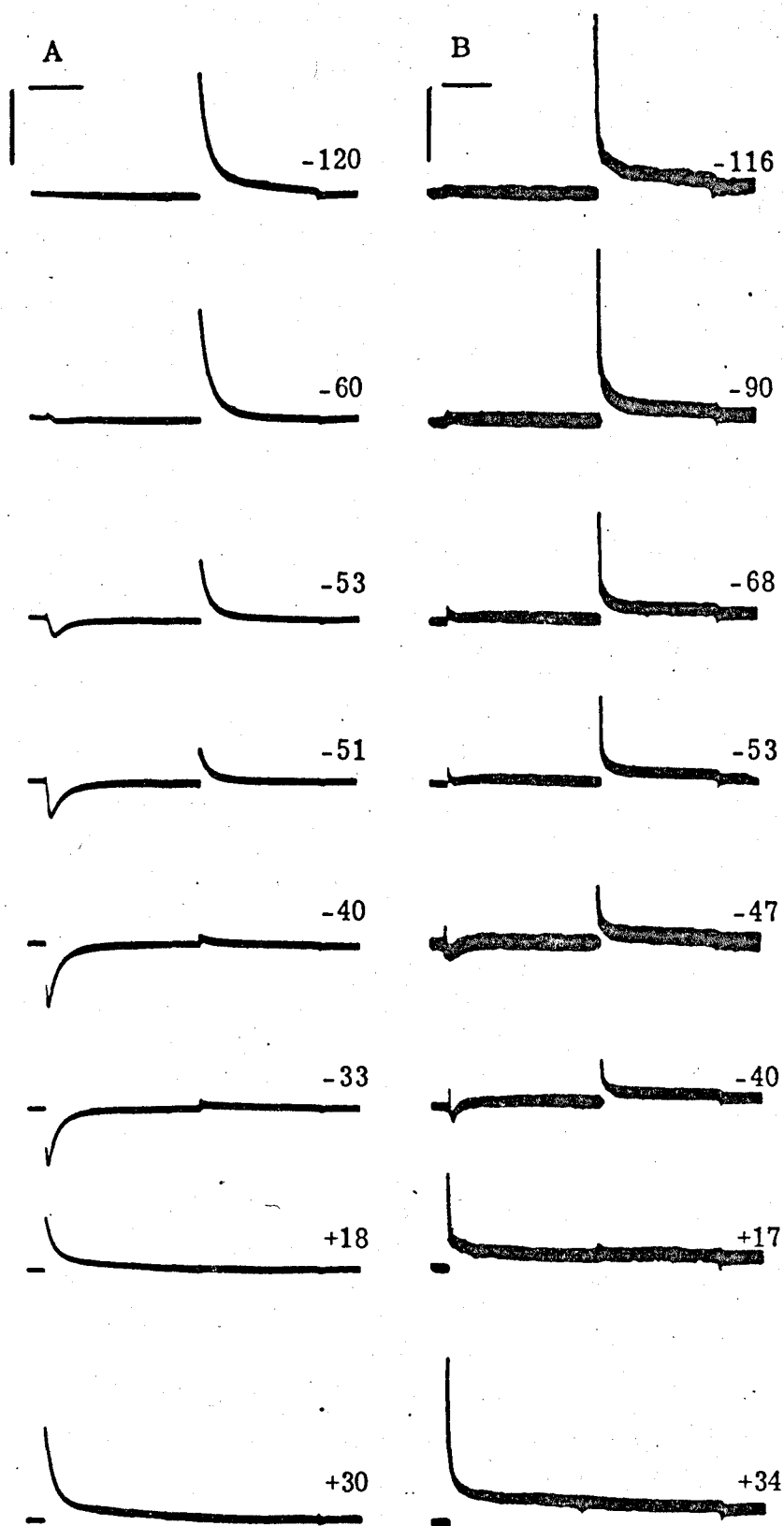


Fig. 16. The effect of 1 mmole/l 4-AP (pH 7.2) on the voltage dependence of n and k . Records A, in the absence of 4-AP; B in the presence of 4-AP. Vertical calibration; A: 0.8 mA/cm²; B: 0.17 mA/cm²; horizontal calibration 200 msec in A and B. V_t in A = +40 mV; and in B = +30 mV. Values for V_c are given in mV beside each trace. Fibre F1 13576: $l=125\mu$; $l'=50\mu$; $a=90\mu$; Temp. 21.5°C.

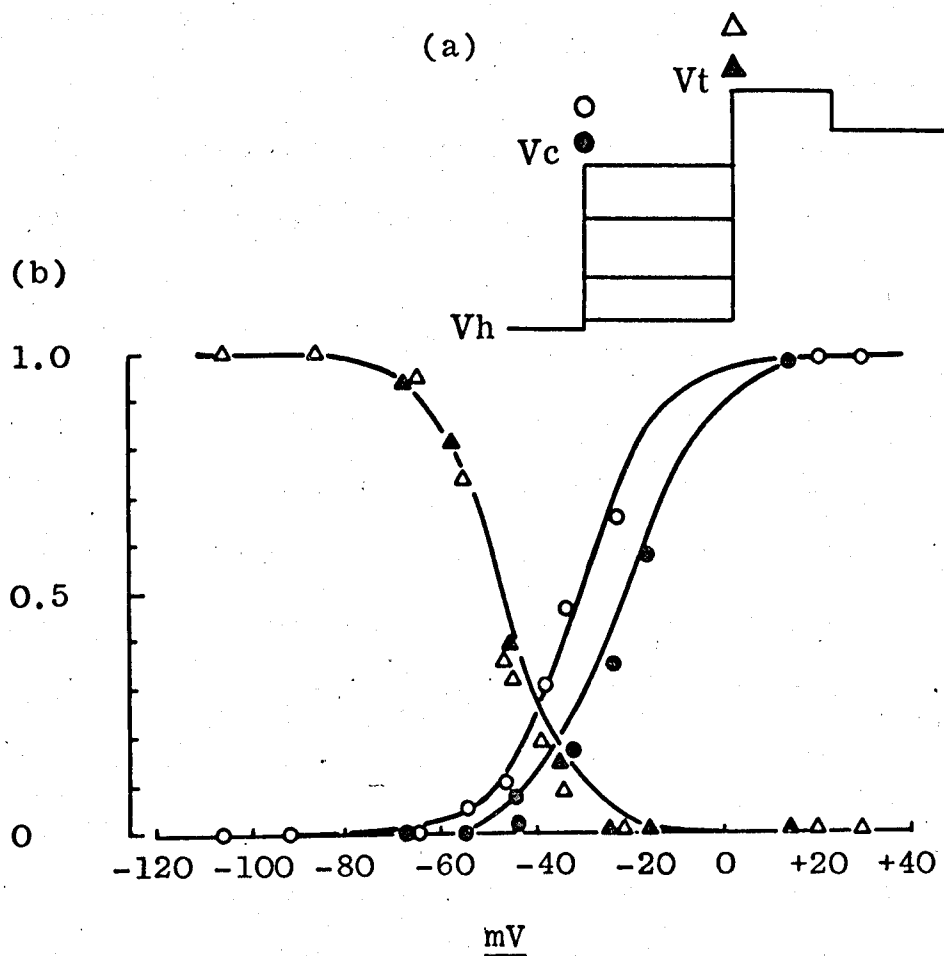


Fig. 17. The voltage dependence of n and k in the absence ($\circ\Delta$) and presence ($\bullet\blacktriangle$) of 1 mmole/l 4-AP (pH 7.2). (a) schematic diagram of the voltage protocol used; (b) a plot of the data shown in Fig. 16: in each condition the data has been normalized to the maximum available potassium conductance. The solid curve through the points describing the steady state inactivation is drawn according to equation I₁₁; the curve through the points describing the normal activation are drawn according to equation III₁ and the curve through the activation points in the presence of 4-AP according to equation III₁ modified by equation III₃₄.

symbols and after 4-AP, filled symbols. The peak conductance during the first depolarizing step (o, ●) and during the second (Δ , \blacktriangle) have been normalized to the maximum available conductance. The activation curve in the presence of 4-AP (●) is shifted in the depolarizing direction in this case by 11 mV whilst the position of the inactivation curve (\blacktriangle), is not altered. This result suggests that the shift in the voltage dependence of the peak current is not due to an effect of 4-AP on the surface potential since a similar shift would then be expected in the inactivation curve. It is more likely that the now 'apparent' shift in threshold is a consequence of the way in which 4-AP blocks the channel.

The actions of 4-AP at different extracellular pH

As discussed earlier, when a weak base such as 4-AP is applied on the outside of the membrane at neutral pH it will become distributed across the membrane such that the concentration of the ionized form on each side of the membrane is equal, $[4\text{-AP}^+_i] = [4\text{-AP}^+_o]$; and the unionized concentrations are also equal, $[4\text{-AP}_i] = [4\text{-AP}_o]$. In order to determine which class of the weak base is responsible for the blocking action experiments were done at different extracellular pH and the results analyzed as previously described.

Fig. 18 shows the results from 3 experiments which follow the action of 1 mmole/l 4-AP at extracellular pH 5.0, 7.2 and 9.8. Each section shows the progressive actions of 4-AP on delayed outward currents in response to a depolarizing step to approximately +30 mV. At each pH, the control current in the absence of 4-AP is shown on the left at $t = 0$ and the remaining traces were recorded at the times indicated after the addition of 4-AP. The delayed rectifier continues to function at these extremes of pH as will be shown in section III_{viii}.

At pH 5.0 1 mmole/l 4-AP reduces the magnitude of the peak current by approximately 50%; there is little change in the time to peak and the time course of inactivation is little altered. In neutral solution

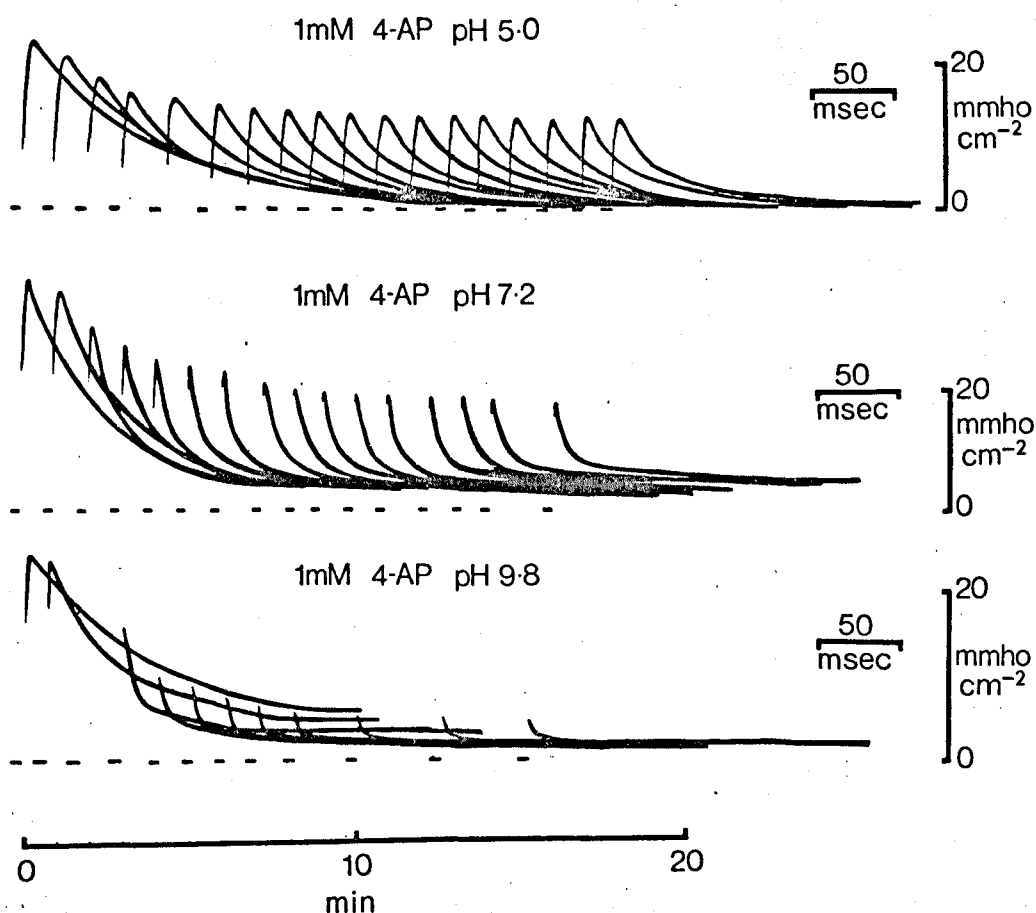


Fig. 18. The effect of 1 mmole/l 4-AP in solution of different pH on delayed outward potassium currents. Each current record was recorded at a sweep speed indicated by the horizontal calibration bar in each section; and recorded at the time indicated after the addition of 4-AP to the bathing solution by the lower time scale. The control record, in the absence of 4-AP is shown at $t=0$ min. At pH 5.0: Fibre 23475: $V_t = +35$ mV; $l=200\mu$; $l'=50\mu$; $a=70\mu$; Temp. 21°C . At pH 7.2: Fibre 25975: $V_t = +30$ mV; $l=200\mu$; $l'=50\mu$; $a=90\mu$; Temp. 21°C . At pH 9.8: Fibre 26117511: $V_t = +30$ mV; $l=250\mu$; $l'=50\mu$; $a=80\mu$; Temp. 19°C .

the result of this experiment is similar to that shown in Fig. 10, however, in this fibre the peak conductance is reduced by 50%. In alkaline solution the peak conductance is reduced to approximately 20%, the time to peak is markedly reduced and the time course of inactivation consists of a more pronounced initial fast component and a later slow component.

In acid solution, the 4-AP is present as 0.9999 mM ionized and only 0.00001 mM non ionized. It is therefore likely that it is the ionized form which is responsible for the blocking action. If the ionized form penetrates the fibre slowly then the action of 4-AP on the delayed current in acid solution may be attributed to the cationic form extracellular solution (4-AP^+_o). At pH 7.2, 4-AP will be distributed across the membrane such that the internal and external concentration of ionized base is 0.99 mM; and the concentration of the non ionized is 0.001 mM. Since the external concentration of ionized base at pH 7.2 is similar to that at pH 5.0, then a major part of the reduction in peak conductance at pH 7.2 may be attributed to the cationic form in the extracellular solution. The decrease in the time to peak and the alteration in the time course of inactivation may therefore be due to the base inside the cell. In alkaline solutions the concentration of ionized base outside the cell will decrease whilst the concentration of ionized base inside, (4-AP^+_i), will increase markedly; consequently any action of the cationic form inside the cell will be accentuated.

The effect of different 4-AP concentrations at pH 7.2 on the peak potassium conductance is shown in Fig. 19. The ratio of the peak conductance before and after the addition of 4-AP is plotted against the log of the 4-AP concentration. For concentrations greater than 0.1 mM the data can be fitted by a curve which describes the binding of one 4-AP molecule to a single blocking site and which has a dissociation constant of 1.1 mM. However, at concentrations less than 0.1 mM the peak conductance in some fibres is increased by 5 to 10% above the untreated value. This increase in peak conductance is probably the same as that transiently observed when following

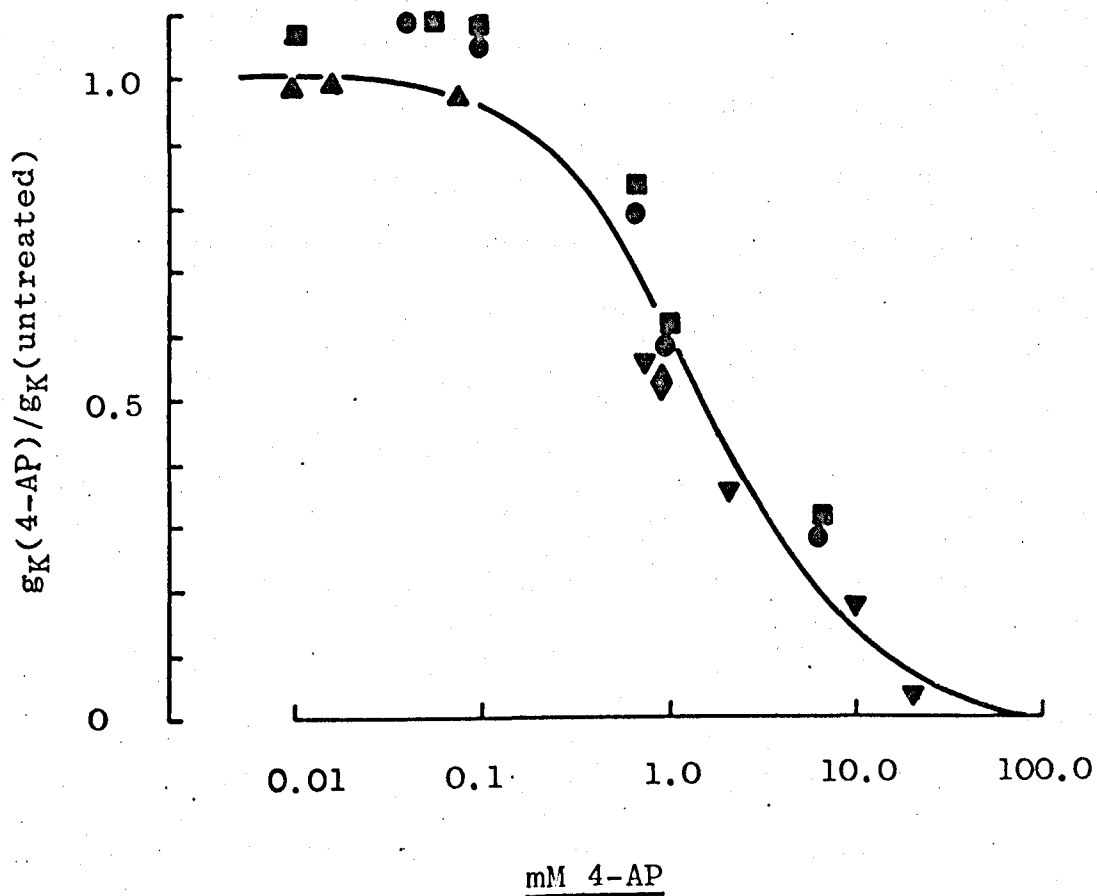


Fig. 19. The effect of different extracellular concentrations of 4-AP on the peak potassium conductance. Solid curve drawn according to equation III₁₃: $K = 1.1$ mM. Data from 4 different fibres; Temp. $20 - 22^{\circ}\text{C}$.

the time course of block by 1 mM 4-AP.

Fig. 20 shows the effect of different concentrations of 4-AP for experiments done in acid and alkaline solution. In acid, the response can be described by a simple chemical reaction with a dissociation constant of 1.0 mM. In alkaline solution the dose response curve is shifted to the left; the dissociation constant is 0.4 mM.

From Fig. 19 and 20 it is possible to construct an ED_{50} - pH relationship for 4-AP and this is shown in Fig. 21. This relation can then be compared with the calculated relationships for each of the possible active forms of 4-AP, shown in Fig. 6. The experimental curve is horizontal in the range pH 5.0 - 7.2, which suggests that $4-AP^+_o$ is the active form; however, the curve falls in alkaline pH which is contrary to what is expected if $4-AP^+_o$ was the only active form. The experimental curve can be fitted if it is assumed that there are two active forms; the cationic form outside the cell, $4-AP^+_o$; and either the ionized or non-ionized form inside the cell. 4-AP inside the cell induces the initial faster inactivation, the rate of which depends upon the membrane potential. It is difficult to imagine how an electric field could influence a non charged molecule therefore it is likely that $4-AP^+_i$ is the second active form.

The effect of TEA^+_o on the formaldehyde treated delayed rectifier

At this point it is of some interest to compare the blocking actions of 4-aminopyridine with those of externally applied Tetraethylammonium ions the more commonly used blocking agent for K^+ current, on this formaldehyde treated preparation. Fig. 22 shows the progressive effect of 5 mM TEA^+_o on outward delayed potassium current recording during depolarization to +32 mV. After the addition of TEA^+ to the bathing solution the peak potassium conductance is reduced: within approximately 15 min the peak conductance is reduced by 48%. Neither the time to peak nor the time course of inactivation is altered by TEA^+_o . The mechanism by which TEA^+_o blocks the potassium channel in muscle

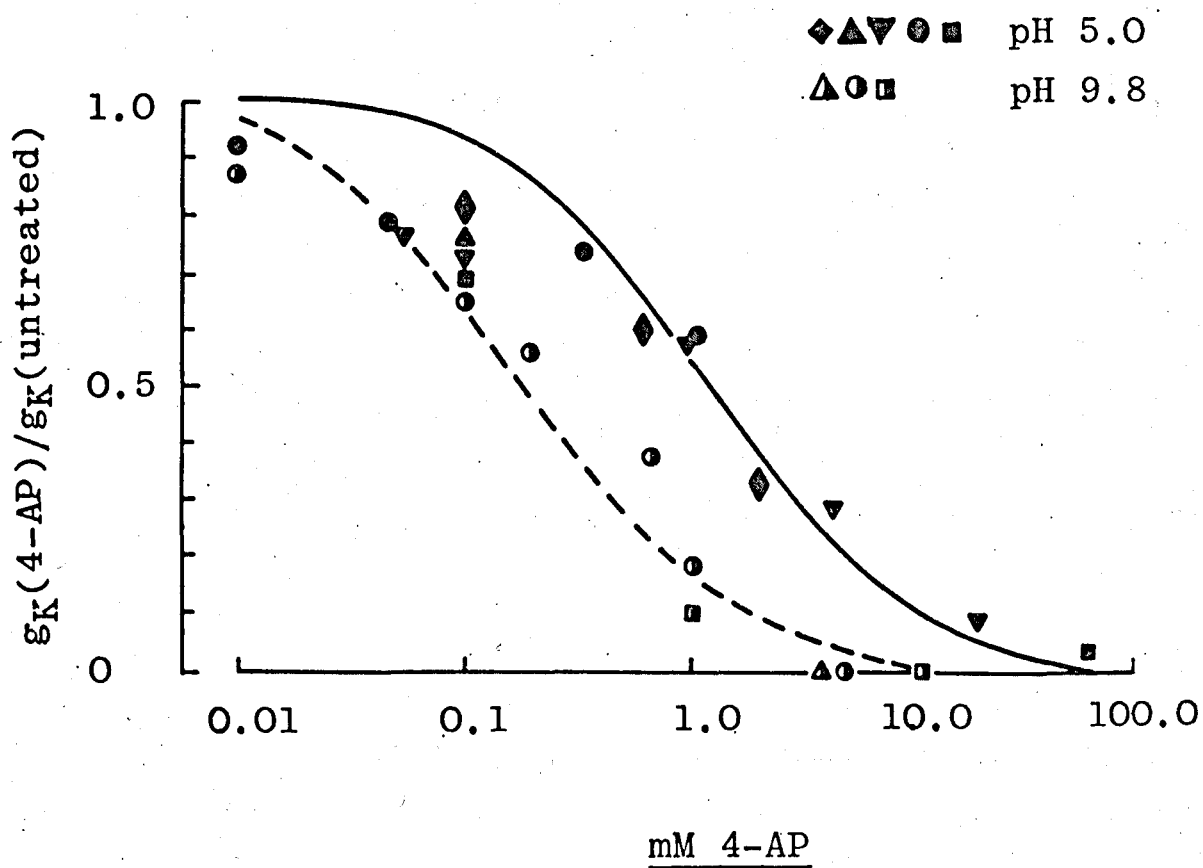


Fig. 20. The effect of different extracellular concentrations of 4-AP at extracellular pH 5.0 and 9.8 on the peak potassium conductance. Curves drawn according to equation III₁₃ with $K = 1.0$ mM and 0.4 mM at pH 5.0 and 9.8 respectively. Data from 8 different fibres; Temp. $20 - 22^{\circ}\text{C}$.

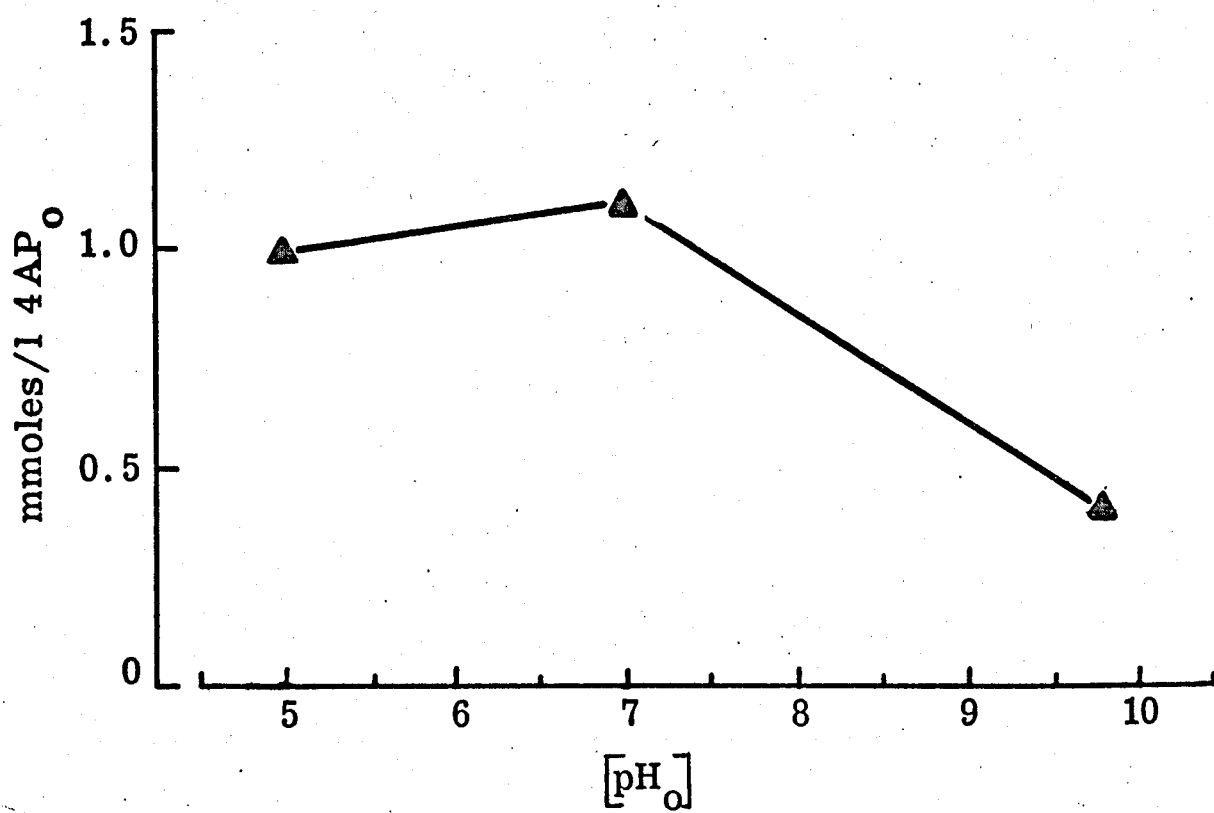


Fig. 21. Experimentally determined ED₅₀— pH relationship for externally applied 4-AP. Points from Figs. 19 and 20.

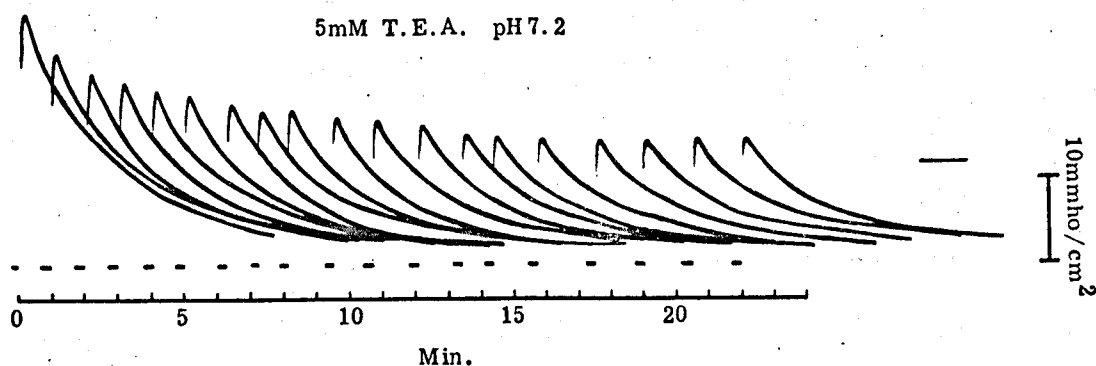


Fig. 22. The effect of 5 mmole/l TEA (pH 7.2) on delayed outward potassium current. Each record was recorded at a speed related to the horizontal calibration bar of 30 msec and at the time indicated on the lower scale after the addition of TEA to the bathing solution. Control record in the absence of TEA shown at t=0 min. Fibre 23455 II: $V_1 = +35$ mV; $l=200\mu$; $l'=50\mu$; $a=70\mu$; Temp. 21°C .

at pH 7.2 is different from that of 4-AP at pH 7.2. On the other hand the TEA^+_o block is similar to that attributed to 4-AP^+_o as found in acid solution. It may be tentatively suggested that 4-AP^+_o and TEA^+_o block the delayed rectifier in skeletal muscle by the same mechanism, and that the difference between 4-AP and TEA at pH 7.2 is due to an action of 4-AP^+_i .

Reversability of the actions of 4-AP

Under the present experimental conditions the blocking actions of 4-AP at pH 5.0 are complete after an exposure of approximately 10 min; reversal of the block after 15 min exposure upon washing in 4-AP free solution was slower and was complete after approximately 60 min. In neutral solution the block is complete after approximately 15 min exposure and reversal is slow; in one experiment only 85% recovery in \bar{g}_K was attained after approximately 60 min wash. At pH 9.8 the action of 4-AP is much more rapid but almost irreversible; washing in 4-AP free solution for over 1 hour produced little recovery.

Caution must be taken in interpreting these results. Firstly, all the experiments were done on whole sartorius muscles in which there is bound to be large diffusion delays. Secondly, as will be shown in section III_{iv}, the onset of 4-AP block in hyperpolarized fibres may depend on the frequency of stimulation.

Preliminary Conclusions

From these preliminary experiments it would appear that the cationic form of 4-aminopyridine is capable of blocking the delayed potassium channel when on the outside and inside of the membrane. When the ionized form is on the outside, 4-AP^+_o , the peak potassium conductance is reduced and there is little change in the time to peak or the time course of inactivation. When the ionized form is also on the inside, 4-AP^+_i , the time to peak is reduced; and there is an initial faster component to inactivation. The following

sections examine in greater detail the blocking actions ascribed to 4-AP_i^+ and 4-AP_o^+ .

III:ii. Effects of 4-AP_i^+ on the delayed potassium conductance

Modifications to the Time Course of Inactivation

Fig. 9 shows a family of delayed potassium currents recorded in the absence of 4-AP. A feature of these untreated currents is that they are not maintained as in nerve but decay with an exponential time constant to zero. Fig. 23 shows a semilogarithmic plot of the change in membrane conductance with time for a fibre depolarized to (a) +30 mV and (b) -30 mV. In each case the decay of current is exponential and proceeds at a rate of 0.0053 msec^{-1} at +30 mV and 0.0049 msec^{-1} at -30 mV. This result is similar to that previously described by Argibay (1974) for such formaldehyde treated fibres.

In the presence of 1 mmole/l 4-AP (pH 7.2) \bar{g}_K is reduced by approximately 50% and in addition the time course of inactivation is no longer a single exponential decay, but consists of an initial fast and later slow component, as can be seen from the family of currents shown in Fig. 11. When the change in membrane conductance for a fibre in the presence of 1 mmole/l 4-AP during depolarizing steps to (a) at +30 mV and (b) -25 mV, is plotted semilogarithmically as in Fig. 24, these two components can be identified. The slow component dominates the decay at times greater than approximately 175 msec, and decays exponentially with a rate constant $(T_{b2})^{-1}$ of 0.0053 msec^{-1} at +30 mV and 0.0048 msec^{-1} at -25 mV. The fast component can be isolated by extrapolating the slow component back to zero time and subtracting the values obtained from the total conductance. Using this procedure it is found that the fast decay is also exponential in this range and has a rate constant $(T_{b1})^{-1}$ of 0.05 msec^{-1} at +30 mV and 0.032 msec^{-1} at -25 mV. The overall time course of inactivation of the delayed current in the presence of 4-AP can then be expressed as the sum of two decaying exponential components, such that:

$$(g_K)_t = A \exp(-t/T_{b1}) + B \exp(-t/T_{b2}) \quad 111.2$$

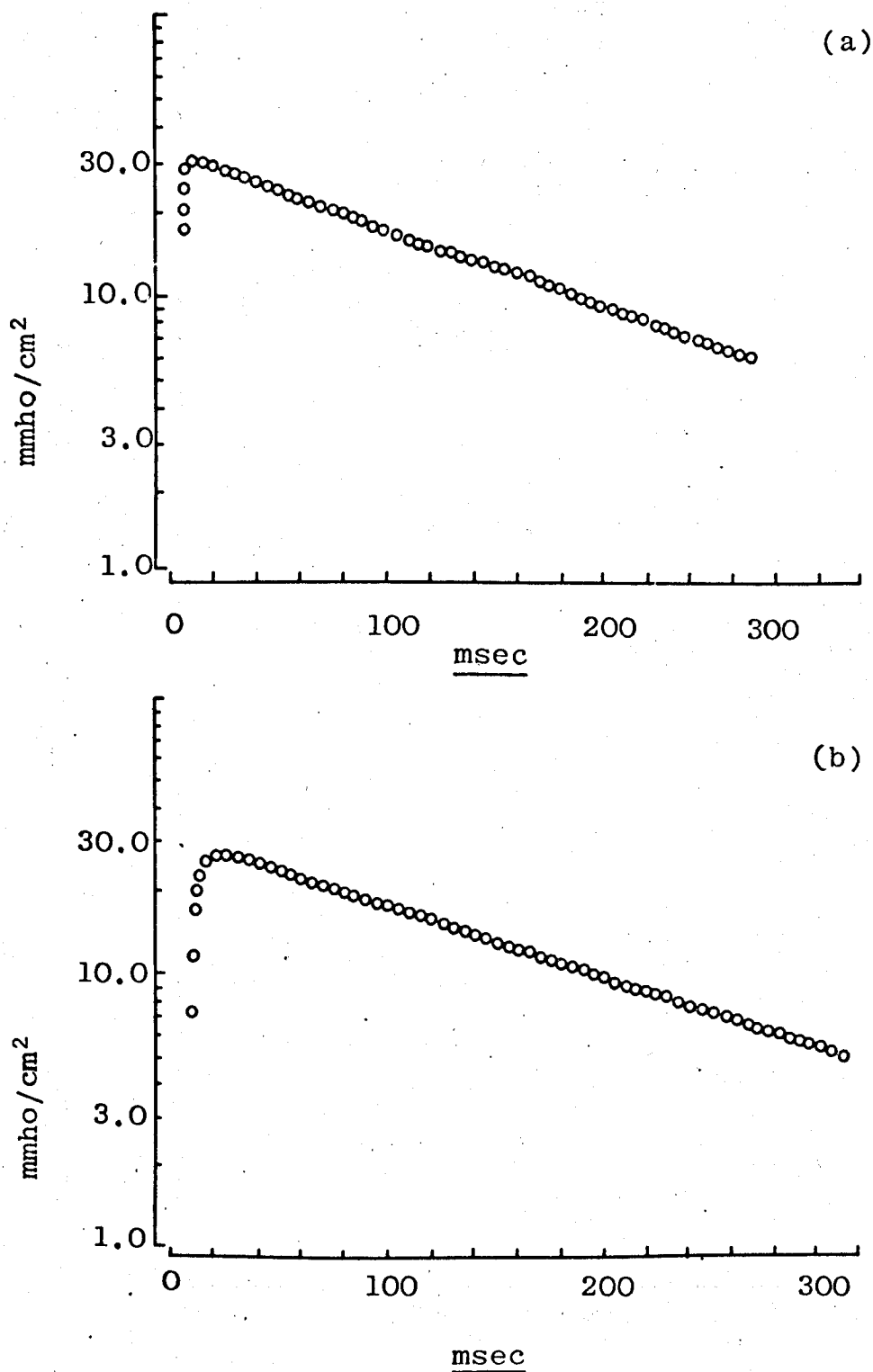


Fig. 23. Semi-logarithmic plot of the change in membrane conductance in the absence of 4-AP (pH 7.2). Fibre 12176 II (a) $V = +30$ mV; (b) -30 mV; $l=250\mu$; $l'=50\mu$; $a=75\mu$; T_k^{-1} in (a) = 0.0053 msec^{-1} , T_k^{-1} in (b) = 0.0049 msec^{-1} . Temp. 21°C .

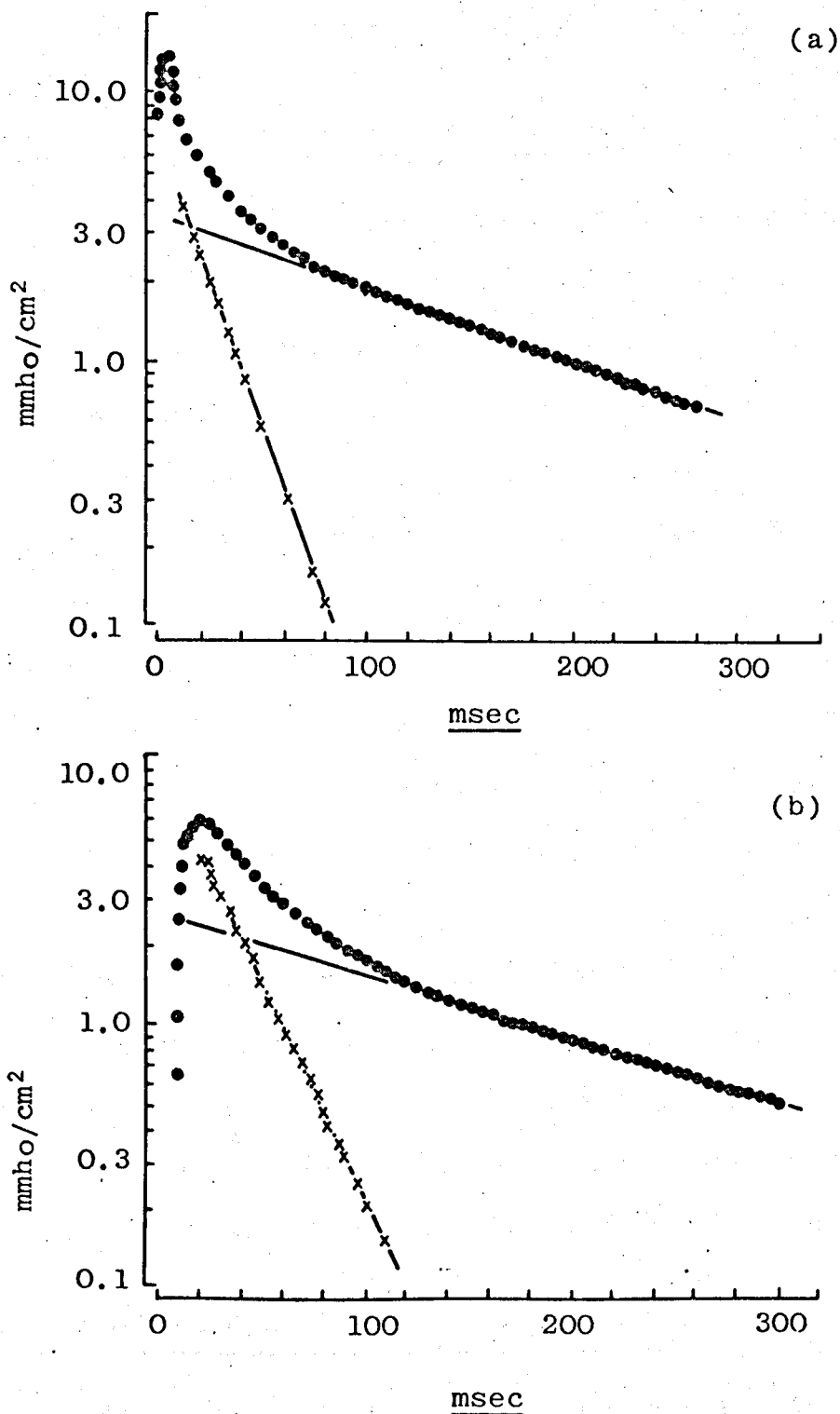


Fig. 24. Semi-logarithmic plot of the change in membrane conductance in the presence of 1 mmole/l 4-AP (pH 7.2). Fibre F2 11076II: (a) $V = +31$ mV, $T_{b1}^{-1} = 0.050$ msec⁻¹, $T_{b2}^{-1} = 0.0053$ msec⁻¹; (b) $V = -20$ mV, $T_{b1}^{-1} = 0.032$ msec⁻¹, $T_{b2}^{-1} = 0.0048$ msec⁻¹; $l = 250\mu$; $l' = 50\mu$; $a = 80\mu$; Temp. 21°C. Straight lines fitted by eye.

where $(g_K)_t$ is the conductance at any time (t); T_{b1} and T_{b2} are the time constants of the fast and slow components respectively; and A and B are the instantaneous conductances of the fast and slow components.

Voltage Dependence of the Rate Constants T_{b1}^{-1} , T_{b2}^{-1} and T_k^{-1} .

The voltage dependence of the rate of inactivation of the untreated current is shown in Fig. 25 (open symbols). In these fibres the rate of inactivation does not systematically depend on potential in the voltage range -50 mV to +80 mV, and has values between 0.004 msec^{-1} to 0.008 msec^{-1} .

Fig. 25 also shows the voltage dependence of the rate of decay of the slow component of inactivation in the presence of 1 mmole/l 4-AP (filled symbols). The slow component does not vary with potential range -50 mV to +80 mV and has values between 0.004 msec^{-1} and 0.008 msec^{-1} . The voltage dependence of the initial fast component (T_{b1}^{-1}) is shown in Fig. 26. The rate constant for this process is dependent on potential in the range -50 to +80 mV and is found to increase as the internal potential is made more positive.

In seeking a working hypothesis to account for these results it may be suggested that the slow component of inactivation in the presence of 4-AP represents a fraction of the channels inactivating normally, thus T_k^{-1} is equal to T_{b2}^{-1} , whilst the initial fast component represents a fraction of the channels that become blocked by 4-AP_i^+ during the depolarizing step.

The Fraction of Available Channels Inactivated by 4-AP_i^+ .

The fraction of available channels that are influenced by 4-AP_i^+ during the course of a depolarizing step may in principal be determined from equation 111.2 using the ratio $A/A+B$. However, in writing equation 111.2 it is assumed that upon depolarization, the rate of inactivation in both processes is achieved instantaneously. Such an assumption may not be valid; each component may inactivate only after an initial delay as is found with the inactivation

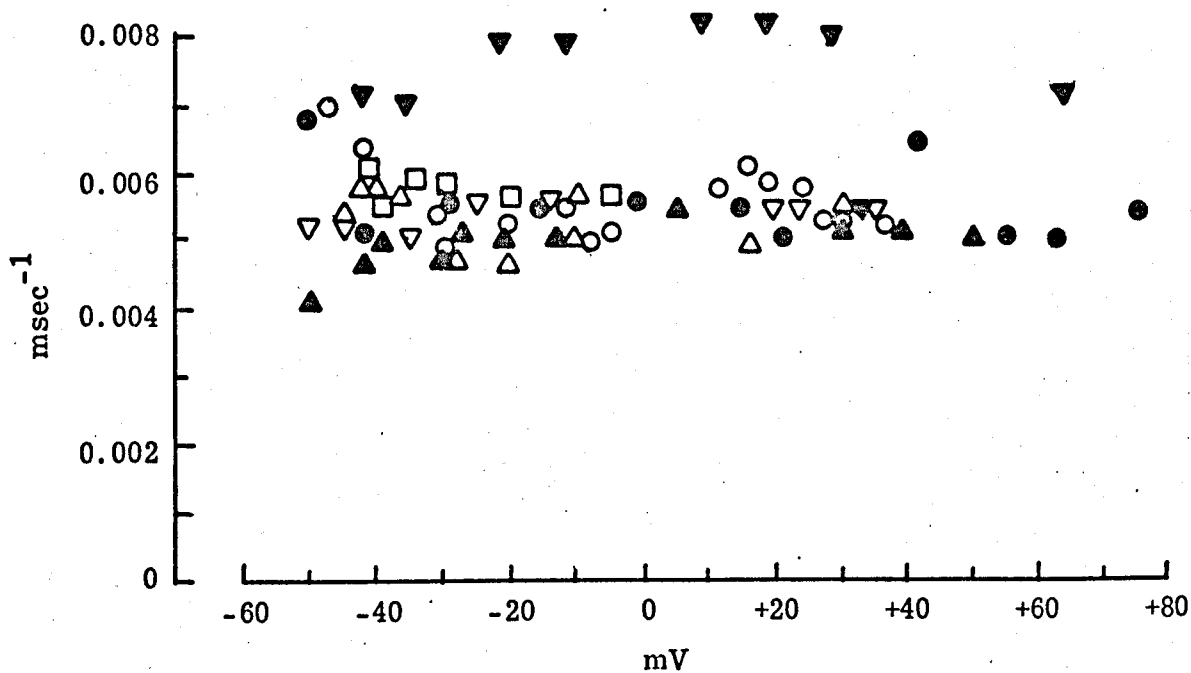


Fig. 25. The rate constant of inactivation in the absence of 4-AP (open symbols) and the slow component of inactivation in the presence of 1 mmole/l 4-AP (pH 7.2) (filled symbols).

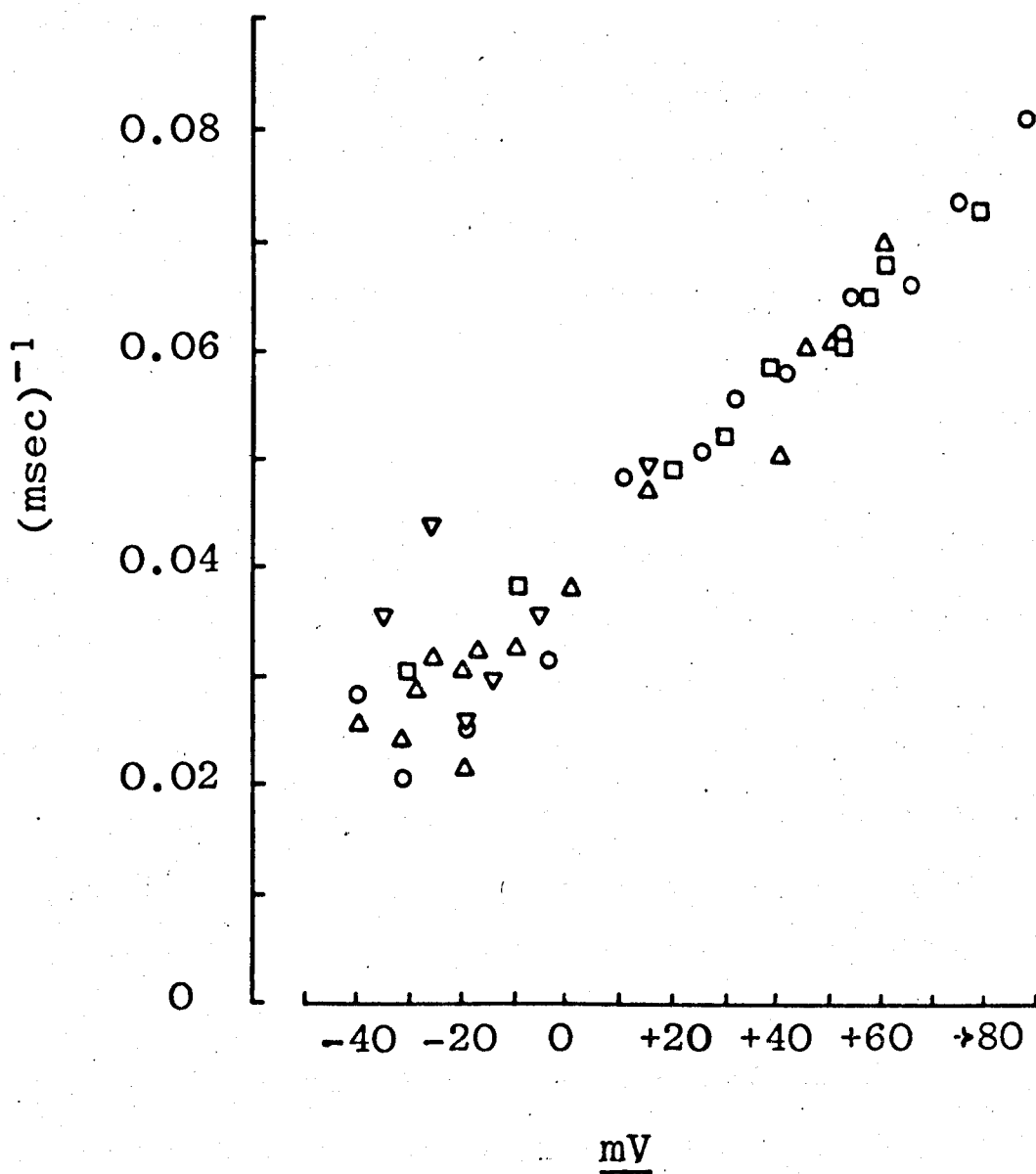


Fig. 26. The voltage dependence of the rate constant of the fast component of inactivation in the presence of 1 mmole/l 4-AP (pH 7.2). Data compiled from 4 fibres; Temp. 20 - 22°C.

of sodium currents in *Myxicola* axons (Goldman, 1975) and with the induced inactivation of potassium channels in squid axon by C_9 (Armstrong, 1967). A delay in either component (T_{b1}) or (T_k) would produce errors when estimating the fraction of channel inactivated from the ratio $A/A+B$. It is necessary therefore to know the exact time course of inactivation of each component in the presence of 4-AP.

Normally the time course of inactivation at the beginning of a depolarizing step is obscured because the conductance is still activating. It is possible however, to determine the time course of inactivation at these early times using a double pulse experiment similar to that devised by Hodgkin & Huxley (1952b).

Whilst the conductance is being activated during a moderate depolarizing step the time course of inactivation at this time can be followed by imposing a second strong depolarizing step after different intervals of time. The peak current envelope of the second test pulse follows the time course of inactivation during the moderate depolarizing pulse. Fig. 28 (a) shows diagrammatically the voltage protocol used in these experiments and Fig. 27 shows examples of the current records obtained in an untreated fibre. From a holding potential of -122 mV the membrane was depolarized to -30 mV which activates a slowly rising inward current. After different intervals of time, (t), a second depolarizing step to +30 mV was given which activated all the residual conductance. In Fig. 28 (b) the logarithm of the peak current is plotted against the duration of the first depolarizing step; the asymptotic value for the decay was obtained at the end of a 3 sec initial depolarizing step as shown in trace A, and was subtracted from all peak values. In the absence of 4-AP, inactivation proceeds exponentially upon depolarization with no indication of an initial delay. This same result was found at all membrane potentials and in all fibres examined. If the same result holds in the presence of 4-AP, then extrapolation of the slow component to zero time will give an accurate value for B.

Fig. 29 shows the current records from a similar experiment done in the

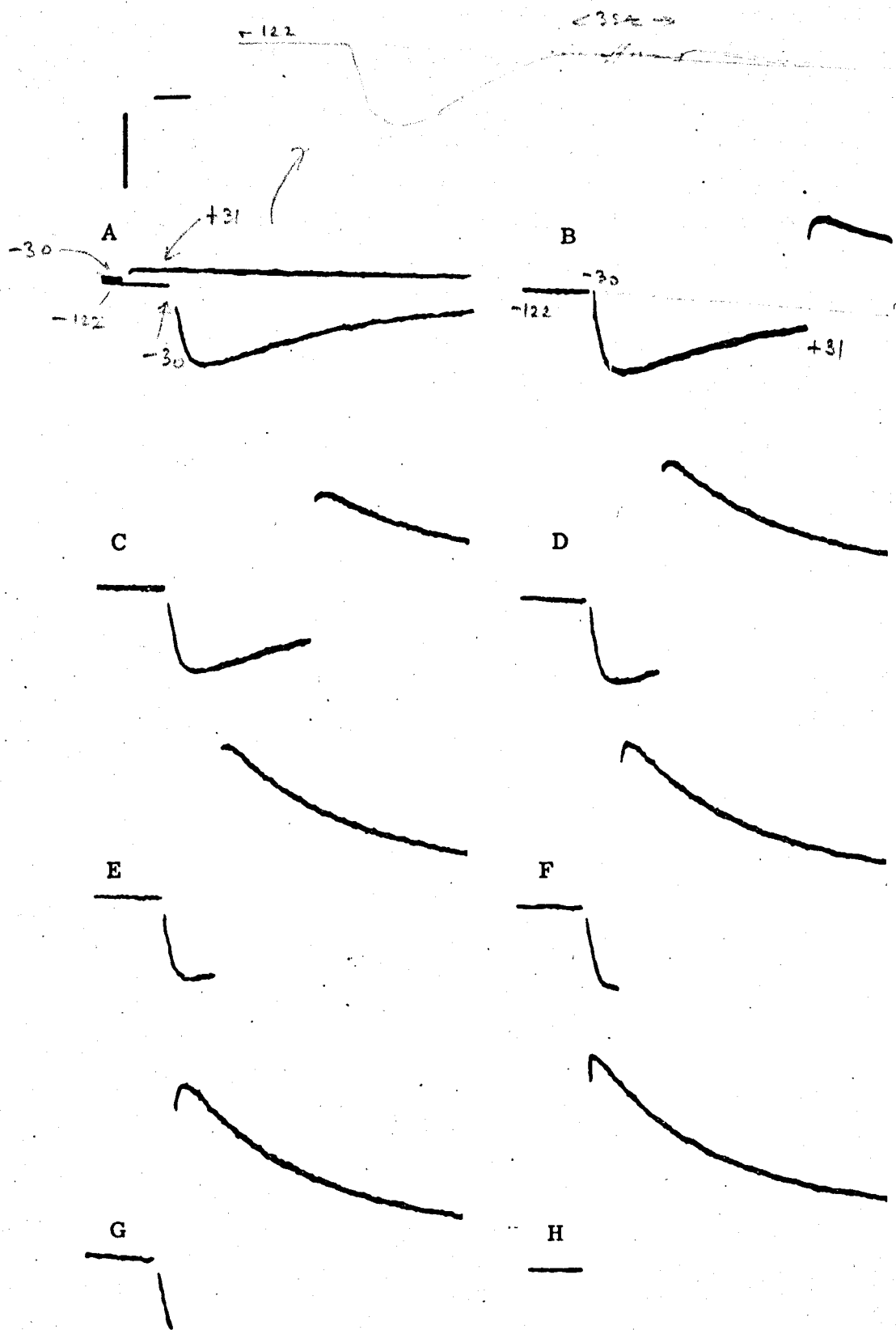


Fig. 27.

Fig. 27. Envelope experiment: records of membrane current in the absence of 4-AP. Record A shows two oscilloscope sweeps; the lower the current flowing at the beginning of a 3 sec conditioning potential (V_c); upper record the peak current activated during the test potential at the end of the 3 sec conditioning pulse. In records B-H, the duration of V_c is made progressively shorter. Horizontal calibration bar: 50 msec; vertical calibration bar: $0.5 \frac{\text{mA}}{\text{cm}^2}$, $V_t = +31 \text{ mV}$; $V_c = -30 \text{ mV}$; $V_h = -122 \text{ mV}$. Fibre F119275 $l = 200\mu$; $l' = 50\mu$; $a = 80\mu$; Temp. 20°C .

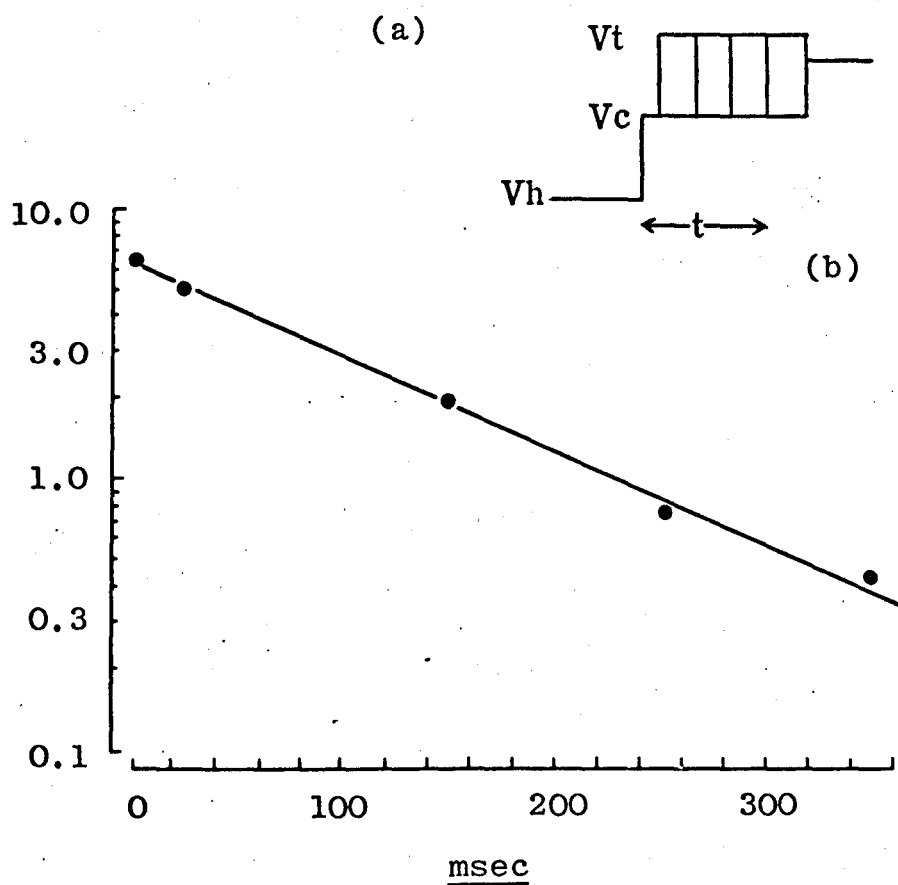


Fig. 28. (a) Diagram of the voltage protocol used in the 'envelope' experiments; V_h : holding potential; V_c : conditioning potential; and V_t : test potential. (b) Semi logarithmic plot of the peak current envelope (minus asymptote) for a fibre in the absence of 4-AP. $V_h = -122$ mV; $V_c = -41$ mV; $V_t = +31$ mV. Fibre F1 19275; $l=200\mu$; $l'=50\mu$; $a=80\mu$. Temp. 20°C .

presence of 1 mM 4-AP; and Fig. 30 (a) shows a semi-log plot from a similar experiment. Two components can be identified in the decay of the peak current envelope, each component can then be extracted as has already been described. The time course of the fast component of inactivation determined thus is not a simple exponential decay, the points at early times are much lower than would expected from extrapolation of the later points to zero. It is possible to check that this "delay" in the 4-AP induced inactivation is not an artifact due to the small number of data points used to derive the value of T_k and T_{bl} . This may be done by comparing the values of T_k and T_{bl} obtained from the peak current envelope to those obtained from the analysis of the conductance change during a single depolarizing step; as shown in Fig. 30 (b). The rate constant of the slow component in (a) is 0.0076 msec^{-1} and in (b) is 0.0066 msec^{-1} ; and the rate constant of the fast component is in (a) 0.033 msec^{-1} and in (b) 0.035 msec^{-1} . Since the rate constant of each component as determined by different methods agree reasonably well it is likely that the initial delay in the time course of inactivation of the fast component is not an error due to the curve fitting procedure and is real.

Table 5 presents the results from 7 such experimental runs. Columns (2) and (3) show respectively the values of A and B determined by extrapolation of the fast and slow component to zero time; column (4) shows the value of $A + B$ as determined from the peak current at $t=0$, from the two step experiment. In every case the measured conductance is less than the extrapolated value, indicating the presence of a delay in the time course of induced inactivation.

The fraction of channels that will be inactivated by 4-AP, (b_∞), in the course of a depolarizing step is therefore given by the expression:

$$b_\infty = \frac{(\bar{g}_K)_{4\text{-AP}} - (B)_{t=0}}{(\bar{g}_K)_{4\text{-AP}}} \quad 111.3$$

where $(\bar{g}_K)_{4\text{-AP}}$ is the maximum potassium conductance in the presence of 4-AP;

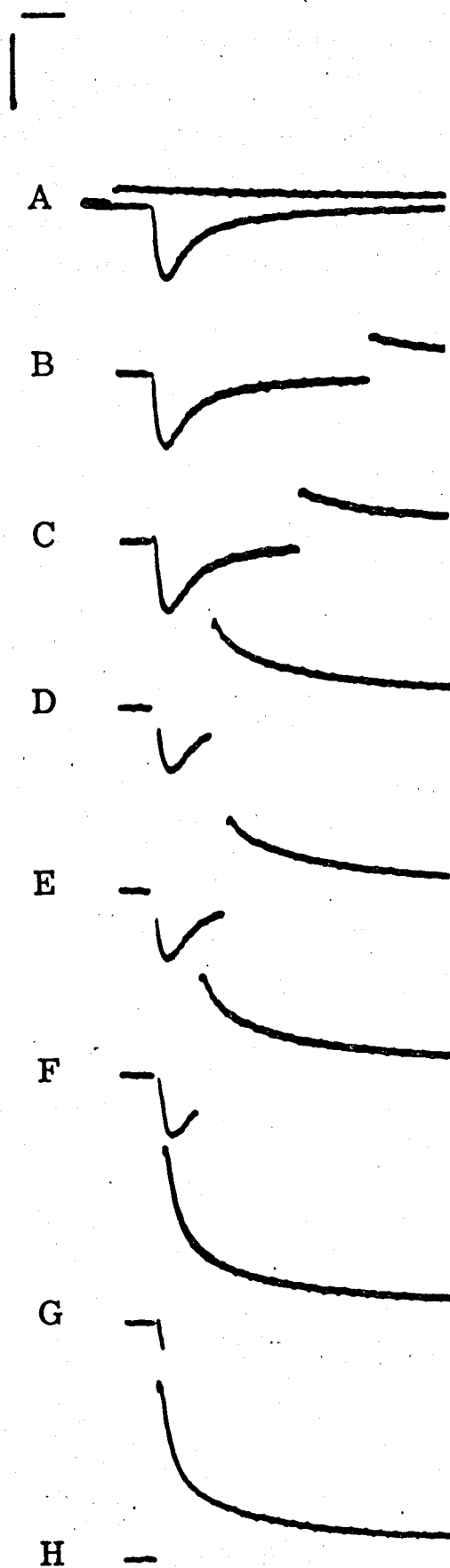


Fig. 29.

Fig. 29. Peak current envelope for a fibre in the presence of 1 mmole/l 4-AP (pH = 7.2). Record A shows two sweeps; lower sweep shows the beginning of a 3 sec step to V_c and the upper shows the end, with a subsequent step to V_t . In records B-H the duration of V_c is progressively reduced. $V_h = -122$ mV; $V_c = -30$ mV; $V_t = +30$ mV. Horizontal calibration bar 50 msec; vertical calibration bar 0.1 mA/cm²: Fibre F310276 II; $l = 250\mu$; $l' = 50\mu$; $a = 80\mu$; Temp. 21°C.

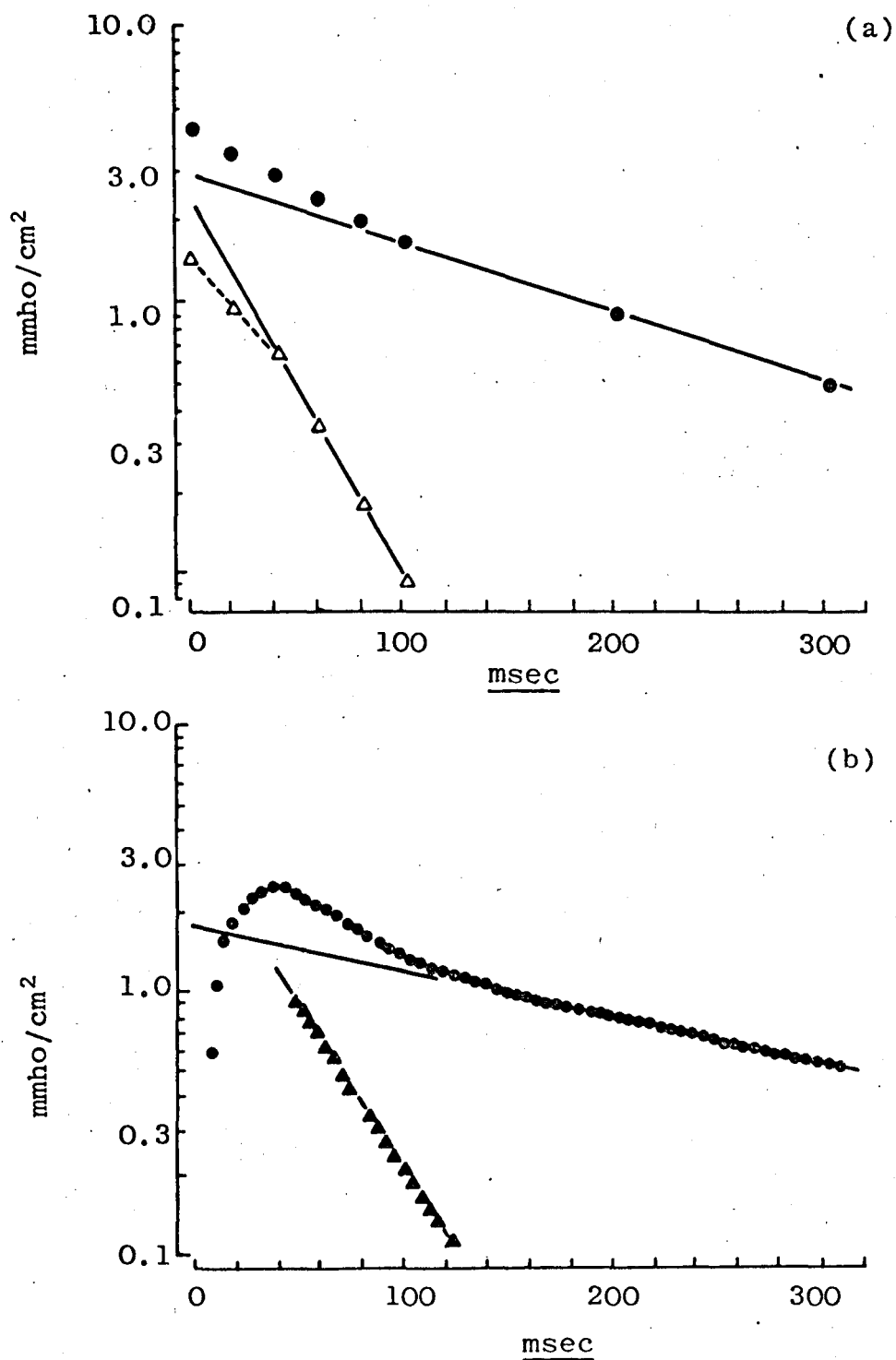


Fig. 30. (a) Semi-logarithmic plot of the peak conductance envelope in the presence of 1 mmole/l 4-AP (pH 7.2). Fibre F2 10276II: $V_c = -30$ mV; $V_t = +31$ mV; $l = 200\mu$; $l' = 50\mu$; $a = 70\mu$; Temp. 21°C . (b) Semi-logarithmic plot of the change in membrane conductance with time during a single depolarizing step. Same fibre as in (a), $V_c = -30$ mV.

	(1)	(2)	(3)	(4)	(5)	(6)	(7)
FIBRE	V_c (mV)	A	B	(A + B)	$(b_o)_{exp}$	b_{∞}	$(b_{\infty})_{est.}$
F3 10276 I	-42	2.5	6.3	8.8	4.4	0.28	0.25
F2 10276 II	-30	1.3	7.7	9.0	7.2	0.55	0.44
F3 10276 II	-16	6.4	3.6	10.0	7.4	0.64	0.67
F3 10276 II	-30	3.2	6.4	9.6	5.6	0.33	0.35
F3 10276 II	-42	4.1	7.5	11.6	6.8	0.35	0.30
F3 06276 II	-36	4.0	7.2	11.2	6.0	0.36	0.36
F4 06276 II	-45	2.4	9.6	12.0	5.4	0.20	-

TABLE 5

The fraction of channels inactivated by 1 mmole/l 4-AP

and $(B)_{t=0}$ is the conductance of the slow component at $t=0$. Column (6) shows the values obtained for b_{∞} .

An approximation to b_{∞} may be got from plots of the membrane conductance during a single depolarizing step by using the expression:

$$b_{\infty} = \frac{(g_K)_{t=p} - (B)_{t=p}}{(g_K)_{t=p}} \quad 111.4$$

where $(g_K)_{t=p}$ is the peak potassium conductance; and $B_{t=p}$ is the conductance of the slow component at the time of peak conductance. Values for the fraction of channels blocked derived from 111.4 above are shown in column (7) and are found to agree well with those shown in column (6). The accuracy of this approximation will increase at more positive potentials, when:

$$(g)_{t=p} \rightarrow (\bar{g}_K)_{4-AP} \quad 111.5$$

and

$$(B)_{t=p} \rightarrow (B)_{t=0} \quad 111.6$$

In the experiment shown in Fig. 29 (a) and (b) the time at which the fast decay becomes exponential coincides with the peak current; that is when all the channels are open. This suggests that $4-AP_i^+$ can only block channels once they are open. A similar delay and a similar interpretation has been reported by Armstrong, 1969 and Armstrong & Hille (1972) to account for their findings on the blocking action of Cg on the potassium channels in squid axons and frog node.

The fraction of the available channels that are inactivated in the course of a depolarizing step in the presence of 1 mmole/l 4-AP (pH 7.2) is shown in Fig. 31. In the voltage range -40 to -10 mV this fraction is strongly dependent on the membrane potential and undergoes an e-fold increase every 12 mV; beyond +10 mV this fraction reaches a limit, $(b_{\infty})_{max}$, which in this case is 0.7. It is noteworthy that the fraction of channels blocked should reach a maximum which is less than unity since the rate at which the

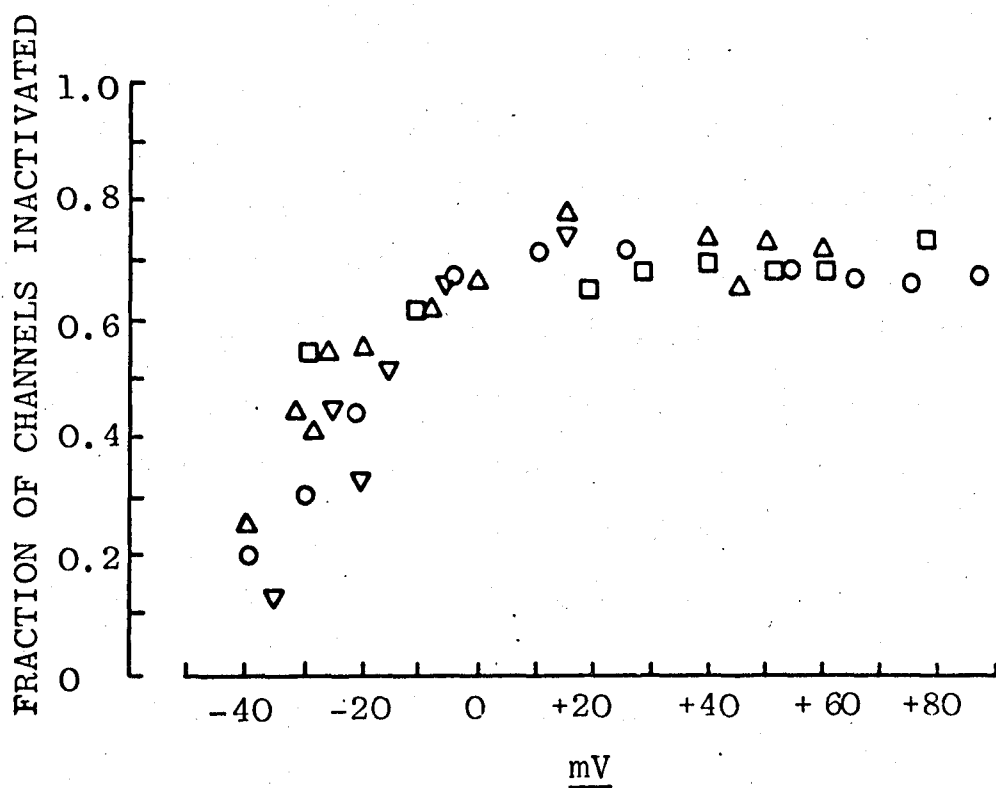


Fig. 31. The voltage dependence of the fraction of channels inactivated in the presence of 1 mmole/l 4-AP (pH 7.2). Temp. 20 - 22°C.

channels are inactivated continues to increase in the voltage range +10 to +80 mV.

In order to explain such a result it is necessary to suppose either that inactivation only occurs in a proportion of the channels, or alternatively, that at positive potentials, channels blocked by the outward movement of 4-AP^+_i become unblocked as 4-AP^+_i moves through the channel away from the blocking site.

This latter suggestion seems unlikely in view of the results at different pH (Section III_i), and the finding that the time course of inactivation can be described by two exponentially decaying processes with no indication of a component increasing with time. In addition, for the fraction of channels inactivated to remain constant, the ratio of the rate of induced inactivation to the rate of release must remain constant; over the potential range, +10 to +80 mV, such constancy seems unlikely. It is more likely that 4-AP is only blocking a proportion of the available channels.

Inactivation induced by different concentrations of 4-AP

The value of the limit to the fraction of channels inactivated, $(b_\infty)_{\text{max}}$, was found to vary with the concentration of 4-AP; this relationship is shown in Fig. 32. As the concentration of 4-AP is increased the limit to the fraction of channels inactivated approaches unity. Such a result would support the idea that 4-AP is binding to a site near the inner mouth of the channel prior to its movement into the blocking position.

The rate constant of the fast component of inactivation determined at +30 mV was also found to vary with the concentration of 4-AP. Fig. 33 shows that as the concentration of 4-AP is increased the rate of inactivation also increases and appears to approach a maximum value of 0.1 msec^{-1} at concentrations greater than 10 mM.

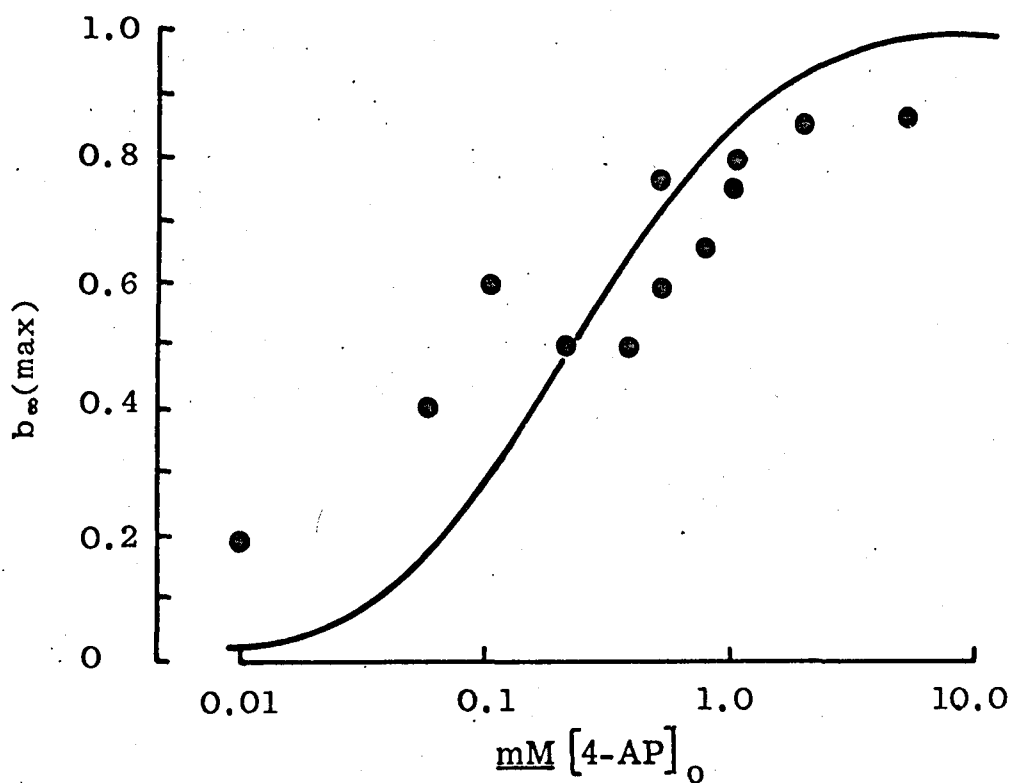


Fig. 32. The effect of different extracellular concentrations of 4-AP on the limit to the fraction of channels inactivated $b_{\infty}(\max)$. Solid curve is drawn according to equation III₁₄; with $K = 0.4$ mM. Data from 8 fibres.

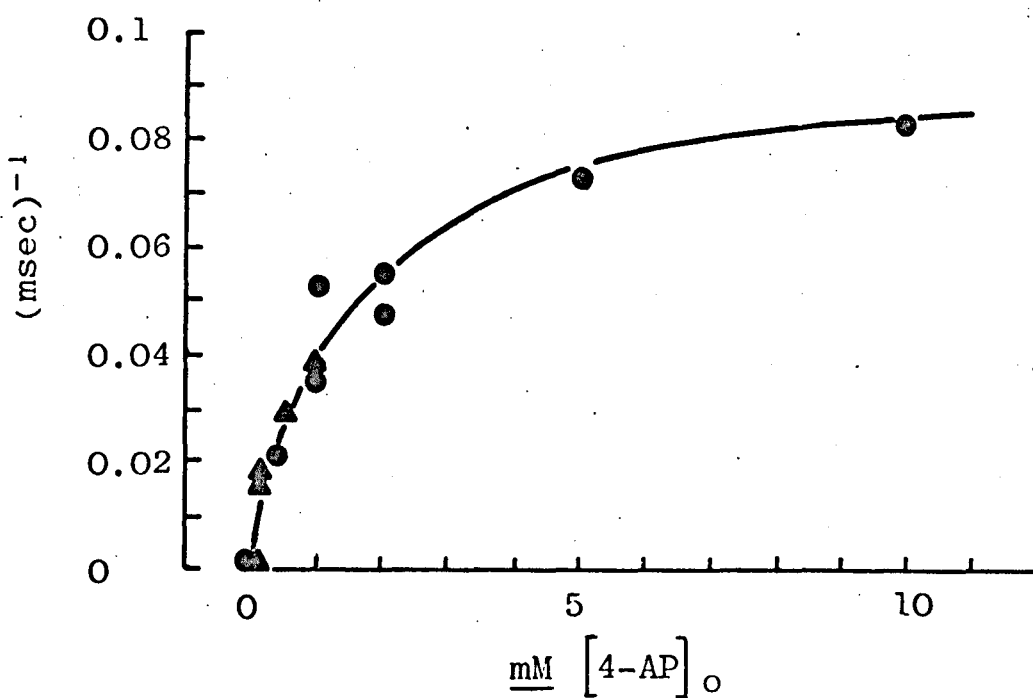
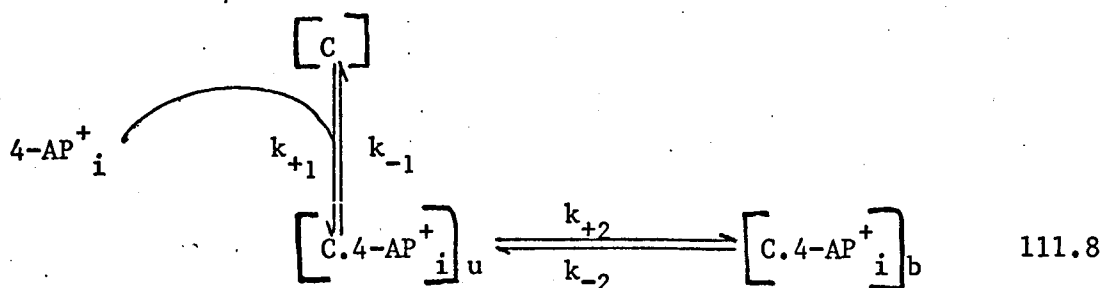


Fig. 33. The rate of induced inactivation during a depolarizing step to +30 mV at different extracellular concentrations of 4-AP (pH 7.2). Solid curve drawn according to equation III₀; $K = 0.1 \text{ mM}$; $(T_b)_{\text{max}}^{-1} = 0.1 \text{ msec}^{-1}$. Temp. 21°C .

Initially both occupied and unoccupied channels are in the conducting state, but with time 4-AP_i^+ , associated with the occupied channels moves into a blocking position C_b^* and the channel ceases to conduct. This block proceeds with rate constants k_{+2} and k_{-2} . In this scheme no account is taken of the 'normal' inactivation (τ_k^{-1}), which proceeds exponentially in all states upon depolarization.

The question arises as to whether the transition of 4-AP_i^+ from the non blocking position to the blocking position is dependent on the membrane potential or the flow of current through the channel. There is some evidence to suggest that the transition is voltage dependent. Firstly, the initial faster component of inactivation is apparent when the potassium current is flowing in the inward and in the outward direction. This is not in keeping with a current dependent reaction where the block by 4-AP_i^+ would be expected to occur only with outward current. Secondly, double pulse experiments which can follow the time course of inactivation during a depolarizing step to the potassium equilibrium potential, when no current flows, still show the presence of induced inactivation. The transition between unblocked and blocked channels appears to be dependent on potential, consequently it may be described by a voltage dependent reaction with forward and reverse rate constants k_{+2} and k_{-2} .

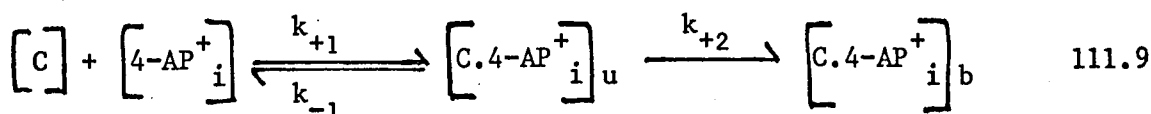
If the channels are assumed to open more quickly than they become inactivated then the reaction scheme 111.7 can be reduced to a much simpler and more easily solved scheme to describe the blockage of occupied channels, and this is outlined below.



where $[C.4\text{-AP}_i^+]_u$ are occupied but unblocked channels and $[C]$ unoccupied

channels respectively; the proportion of channels in each state is governed by k_{+1} , k_{-1} and $[4\text{-AP}^+_i]$. Upon depolarization all the channels, $[C] + [C.4\text{-AP}^+_i]_u$, become conducting, but the occupied channels become blocked at a rate and to an extent determined by k_{+2} and k_{-2} to become $[C.4\text{-AP}^+_i]_b$. Once the internal potential has been made positive only that 4-AP which is bound to the channel is pushed into the membrane. Apparently no 4-AP ions which are free in the intracellular solution move directly into the channel. A possible explanation for this observation is that the binding at the inner surface is also potential dependent. Upon depolarization binding at this site is reduced, consequently no more 4-AP can gain access to the channel. Some evidence for a voltage dependent binding at the surface site is the finding that the binding reaction apparently sees twice the applied electric field (see page 54). In the present analysis it will be assumed that this binding is maximal at the holding potential of -122 mV and depends only on the concentration of 4-AP^+_i .

For the special case where $k_{+2} \gg k_{-2}$ then the scheme becomes



Such a scheme is similar to the sequential reaction scheme of the binding of a substrate to an enzyme and subsequent reaction. The rate of production of $[C.4\text{-AP}^+_i]_b$, i.e. the rate of inactivation, can be described by Michaelis Menton type kinetic and is given by:-

$$(T_b)^{-1} = \frac{(T_b)_{\max}^{-1} [4\text{-AP}^+_i]}{\frac{k_{-1} + k_{+2}}{k_{+1}} + [4\text{-AP}^+_i]} \quad 111.10$$

The rate of the induced inactivation is thus proportional the concentration of 4-AP^+_i and the ratio $\frac{k_{-1} + k_{+2}}{k_{+1}}$. At any given concentration of 4-AP^+_i k_{+1}

and k_{-2} are constant; k_{+2} however, varies with potential, as a result the rate of inactivation will be potential dependent. Under such circumstances

$$T_b = C_1 k_{+2} + C_2 \quad 111.11$$

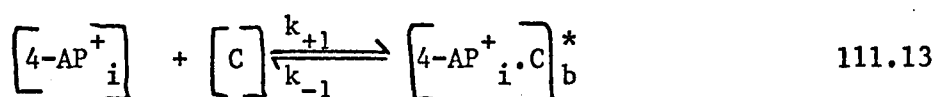
where C_1 and C_2 are constants. The experimentally determined voltage dependence of T_b , as shown in Fig. 26, is directly proportional to the voltage dependence of k_{+2} .

For a given depolarizing step, the value of k_{+2} is constant; in this situation the rate of the induced inactivation will depend only on the concentration of 4-AP_i^+ . Equation 111.10 then becomes:

$$(T_b)^{-1} = \frac{(T_b)_{\max}^{-1} [4\text{-AP}_i^+]}{K + [4\text{-AP}_i^+]} \quad 111.12$$

A plot of $[4\text{-AP}_i^+]$ against T_b^{-1} is thus a hyperbola. The maximum rate is attained when all channels are occupied. The solid curve in Fig. 33 is drawn according to equation 111.12 with $(T_b)_{\max}^{-1} = 0.1 \text{ msec}^{-1}$ and $K = 0.1 \text{ mM}$.

At the end of a long, strong depolarizing step all occupied channels are in the blocked state and the reaction scheme can be simplified further to:



In this situation, the fraction of channels inactivated is at a maximum, $(b_\infty)_{\max}$; it can then be shown that this limit is given by:

$$(b_\infty)_{\max} = \left(\frac{k_{+1}}{k_{-1}} [4\text{-AP}_i^+] + 1 \right)^{-1} \quad 111.14$$

The solid curve in Fig. 32 is drawn according to equation 111.14 with the dissociation constant for the reaction (k_{+1}/k_{-1}) equal to 0.4 mM.

The voltage dependence of the individual rate constants k_{+2} and k_{-2} can be calculated from the voltage dependence of the fraction of occupied channels which are blocked and the voltage dependence of the rate of inactivation

$$(b_{\infty}) = \frac{k_{+2}}{k_{+2} + k_{-2}} \quad 111.15$$

and

$$(T_b) = k_{+2} + k_{-2} \quad 111.16$$

The fraction of occupied channels in the blocked state at each voltage can be obtained after normalizing the data in Fig. 31; this is shown in Fig. 34, k_{+2} and k_{-2} can then be calculated. The voltage dependence of k_{+2} and k_{-2} is shown in Fig. 35. Equations which describe the experimentally determined voltage dependence of k_{+2} and k_{-2} are shown below.

$$k_{+2} = 6 \times 10^{-3} (V - \bar{V}) + 0.016 \quad 111.17$$

and

$$k_{-2} = 0.016 \exp - \frac{(V - \bar{V})}{20} \quad 111.18$$

The dissociation constant for the blocking reaction $\frac{k_{-2}}{k_{+2}}$ is voltage dependent and can also be approximated to and expressed in an alternative form by the relation:

$$\frac{k_{-2}}{k_{+2}} = K_o \exp - (Z\delta EF/RT) \quad 111.19$$

This expression is the same as that derived by Strichartz (1973) (see appendix) to describe the voltage dependent dissociation constant for the blocking reaction of the lidocaine derivative QX314 on the sodium channel in nerve. K_o is the dissociation constant in the absence of an electric field (E); Z is the charge on the reactant, in this case +1; and δ is the fraction of the applied electric field which affects the reaction. At 20°C $\frac{RT}{F}$ is equal to 25 mV. The fraction of channels that are blocked at any membrane potential is given by:

$$(b_{\infty}) = \frac{k_{+2}}{k_{+2} + k_{-2}} = \frac{1}{1 + K_o \exp - (Z\delta EF/RT)} \quad 111.20$$

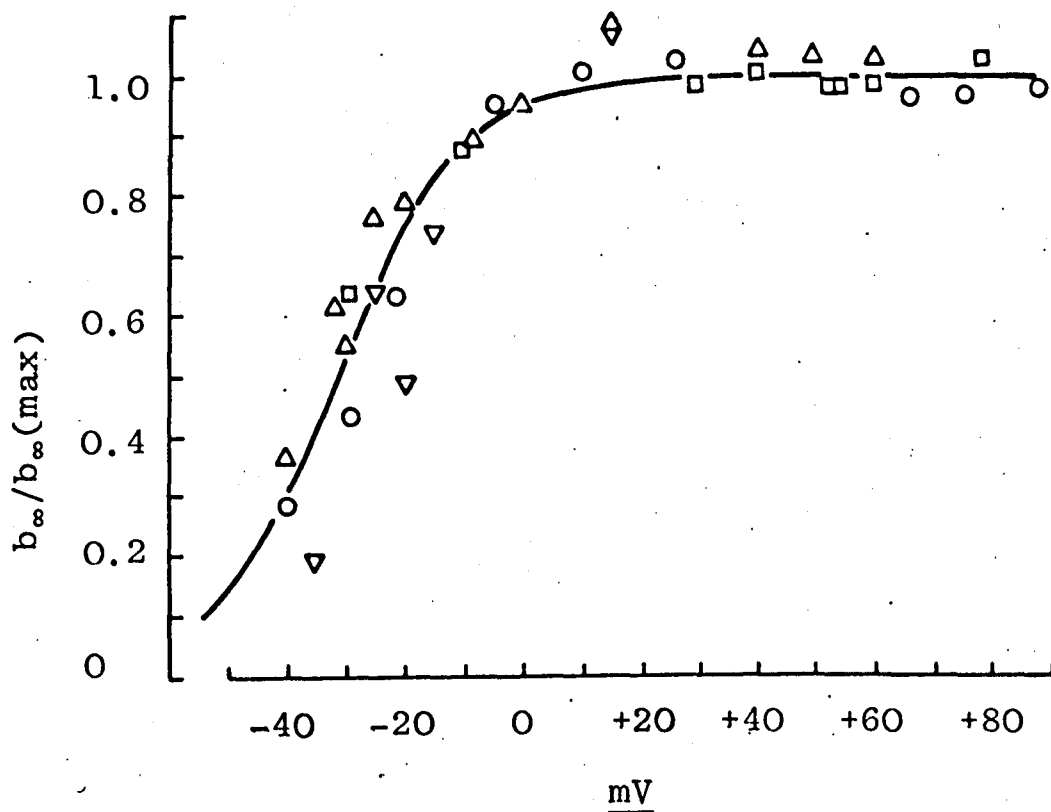


Fig. 34. The voltage dependence of the ratio $b_{\infty}/b_{\infty(\max)}$ for fibres in the presence of 1 mmole/l 4-AP (pH 7.2). Solid curve is drawn according to equation III₁₇; with $\delta = 2.0$.

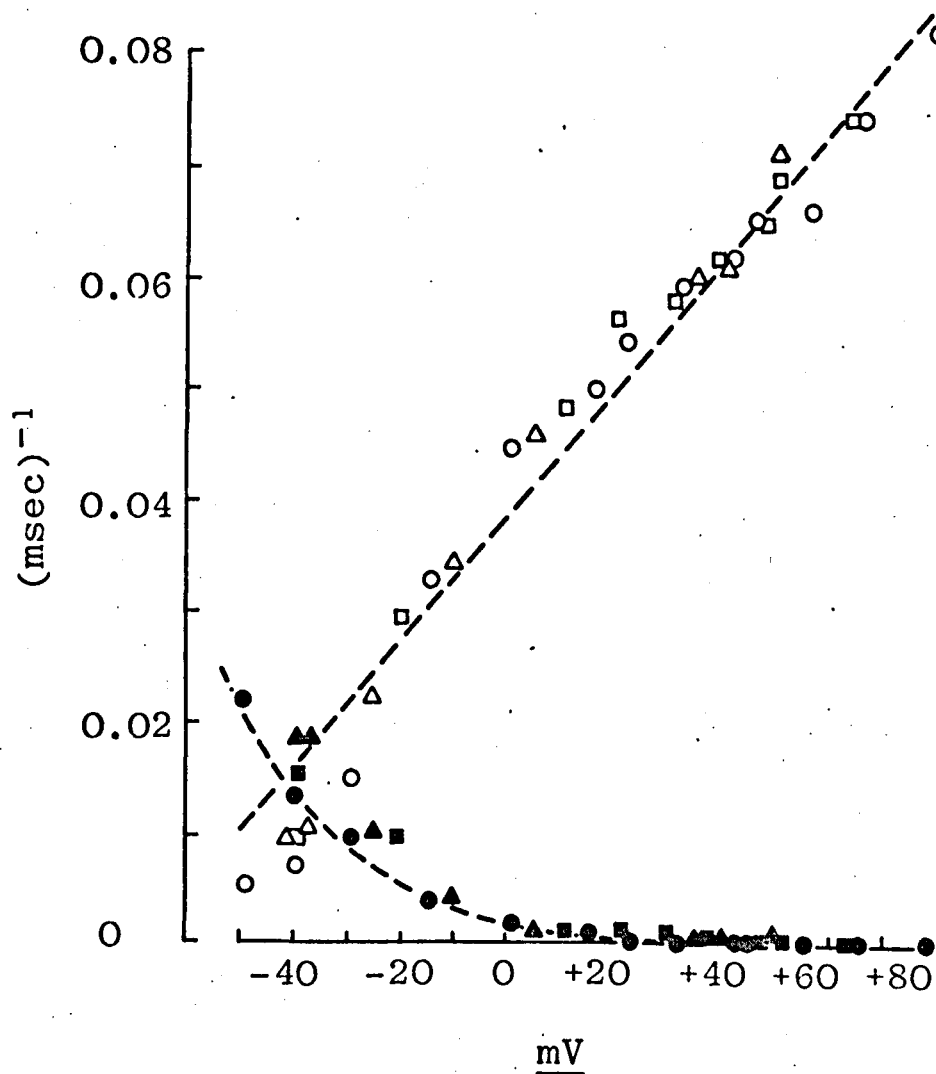


Fig. 35. The voltage dependence of the forward and reverse rate constants k_{+2} and k_{-2} . The curves are drawn according to equation III₁₄ and III₁₅.

The solid curve in Fig. 34 is drawn according to equation 111.20 with a value of δ equal to 2.0. Note that in this case, δ has a value greater than unity and cannot be simply interpreted as the fraction of the applied field acting on the blocking site.

A similar situation, where $\delta > 1$, has been reported by Hagiwara, Miyazaki & Rosenthal (1976) to occur during the voltage dependent blocking by C_s^+ of the inwardly rectifying potassium channels in star fish egg cell membrane. In this case δ equals 1.5. A model for the potassium channel which can account for a voltage sensitive blocking reaction with values of $\delta > 1$ has been described by Hille (1975b) and Hagiwara, Ciani & Miyazaki (1977), and involves two sequential voltage dependent binding reactions. In this model, the channel, or part of it, contains two binding sites, each of which is able to bind either potassium ions (K^+) or the blocking ions (B^+). The channel is blocked only when the site deepest in the membrane, the inner site, is occupied by the blocking ion. Under these circumstances, the expression for the fraction of channels blocked, (R), in the steady state has been derived by Hagiwara et al (1977) and is:-

$$R = \frac{1}{1 + K_B^O [B^+] \exp - \left(\frac{\delta_2 E}{25}\right) + K_B^O K_K^B [B^+] [K_i^+] \exp - \left(\frac{(\delta_2 + \delta_1) E}{25}\right)} \quad 111.21$$

where K_B^O is the binding constant for the blocking ion to the inner site when the outer site is unoccupied; K_K^B is the binding constant for potassium ions to the outer site when the inner site is occupied by B^+ ; and δ_1 and δ_2 are the fraction of the applied electric field seen at the outer and inner sites respectively. If the binding of B^+ to the inner site in an open unoccupied pore was negligible then the fraction of channels blocked is given by

$$R = \frac{1}{1 + K_B^O K_K^B [B^+] [K^+] \exp - \left(\frac{(\delta_2 + \delta_1) E}{25}\right)} \quad 111.22$$

Under these conditions the voltage sensitivity δ' of the overall blocking reaction is $(\delta_2 + \delta_1)$, which may have a value greater than unity.

In order to account for the voltage sensitivity of the steady state block induced by 4-AP, where $\delta = 2$, it may be assumed that there are two binding sites, S_i and S_i' , located close to the inner surface of the membrane. The site nearer the surface of the membrane may be the same site that is occupied by 4-AP_i^+ prior to its movement into the blocking site; and the inner site is the blocking site. At present there is no information on the influence of K_i^+ on the blocking action of 4-AP_i^+ , but an interaction may be anticipated.

The time course of 4-AP_i^+ inactivation at early times

If the 4-AP_i^+ induced inactivation only proceeds in open channels then the rate of inactivation may be assumed to be proportional to the number of channels open. Adapting the Hodgkin Huxley formulation, then

$$(\tau_b')^{-1} \propto (n_t)^4 \quad 111.23$$

where (n_t) is the variable which describes the number of open channels. When all the available channels are open the rate of inactivation reaches a maximum, $(\tau_b)^{-1}$. At all times, the rate of inactivation is then given by:

$$(\tau_b')^{-1} = (\tau_b)^{-1} (n_t)^4 \quad 111.24$$

If $(b)_t$ is the fraction of channels not blocked by 4-AP at time (t) , then $(b)_t$ is given by the expression:

$$(b)_t = \exp - \left(\frac{t (n_t)^4}{\tau_b} \right) \quad 111.25$$

(n_t) is given by the Hodgkin Huxley formulation

$$(n_t) = (n_\infty - (n_\infty - n_0) \exp - \frac{t}{\tau_n}) \quad 111.26$$

In a fully primed fibre $n = 0$, and upon strong depolarization $n = 1$; under these conditions then

$$(n_t) = (1 - \exp \frac{-t}{T_n}) \quad 111.27$$

The change in potassium conductance (g_K) with time in the absence of normal inactivation and 4-AP is given by:-

$$g_K = \bar{g}_K (1 - \exp \frac{-t^4}{T_n^4}) \quad 111.28$$

In the presence of 4-AP, when all the channels are occupied the time course of the conductance change is given by:

$$g_K = \bar{g}_K (1 - \exp \frac{-t^4}{T_n^4}) \cdot (b)_t \quad 111.29$$

$$\Rightarrow \frac{g_K}{\bar{g}_K} = (1 - \exp \frac{-t^4}{T_n^4}) \exp \left(-\frac{t(n)_t^4}{T_b} \right) \quad 111.30$$

$$\Rightarrow \frac{g_K}{\bar{g}_K} = (1 - \exp \frac{-t^4}{T_n^4}) \exp \left(-\frac{t(1 - \exp -t/T_n)^4}{T_b} \right) \quad 111.31$$

Fig. 36 shows a plot of equations 111.26 and 111.29 using values for the constants T_n and T_b for those found experimentally at membrane potentials of +50 mV ($T_n = 1$ msec, $T_b = 10$ msec), and -30 mV ($T_n = 15$ msec, $T_b = 25$ msec). n_∞ is assumed to be unity in each case. Two interesting points arise from these calculated curves.

Firstly, the time to peak of the 4-AP treated current is reduced, despite the fact that T_n is the same. It is not necessary, on the grounds of a decrease in the time to peak, to propose a decrease in T_n by 4-AP. Secondly, the slower rate of activation at -30 mV means that a larger fraction of the channels can be inactivated by the time to maximum conductance i.e. $n = 1$. This situation leads to a larger reduction in the peak conductance at more negative potentials; consequently this may provide an explanation for the

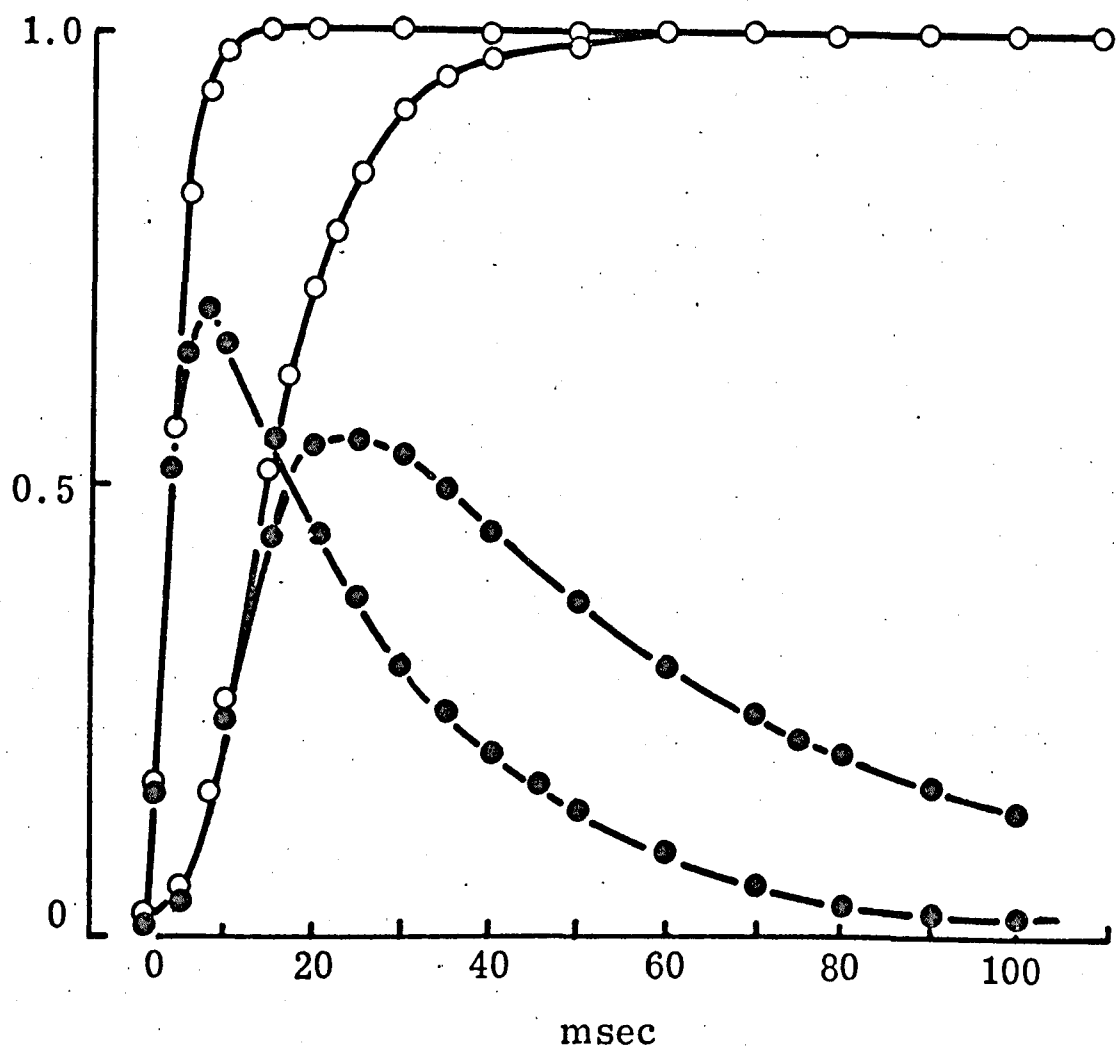


Fig. 36. Plot of equations III₂₆ and III₂₉ for two values of T_n (1 msec and 10 msec). Open symbols: in the absence of normal inactivation ; filled symbols: in the absence of normal inactivation but in the presence of 4-AP. n_∞ set equal to 1 in each case; all channels occupied by 4-AP.

apparent shift in the threshold of activation, as reported in section III_i.

When only a fraction of the channels are occupied by 4-AP⁺_i the time course of the conductance change is given by:

$$\frac{g_K}{g_K} = A \left(1 - \exp \frac{-t^4}{T_n^4}\right) + B \left(1 - \exp \frac{-t^4}{T_n^4}\right) \cdot \exp - \frac{(t(1 - \exp \frac{-t^4}{T_n^4}))}{T_b} \quad 111.32$$

where $A + B = 1$ and, $B = \left(\frac{k+1}{k-1} \left[4-AP_i^+\right] + 1\right)^{-1}$

Taking into account normal inactivation equation 111.32 becomes

$$\frac{g_K}{g_K} = A \left(1 - \exp \frac{-t^4}{T_n^4}\right) \cdot \exp \frac{-t}{T_k} + B \left(1 - \exp \frac{-t^4}{T_n^4}\right) \cdot \exp - \frac{(t(1 - \exp \frac{-t^4}{T_n^4}))}{T_b} \cdot \exp \frac{-t}{T_k} \quad 111.33$$

Fig. 37 shows 3 plots of equation 111.33 under 3 different conditions. The triangles are experimentally derived conductances in the presence of 4-AP multiplied by a scaling factor, and it can be seen that they fit the calculated curves reasonably well.

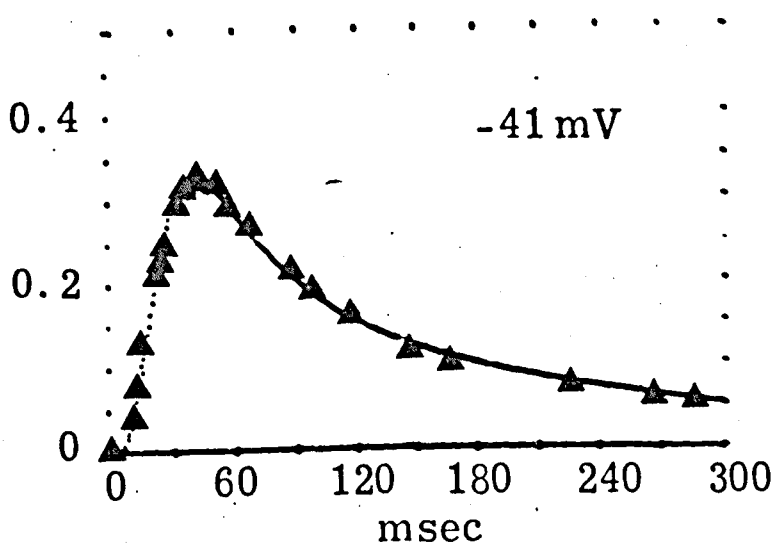
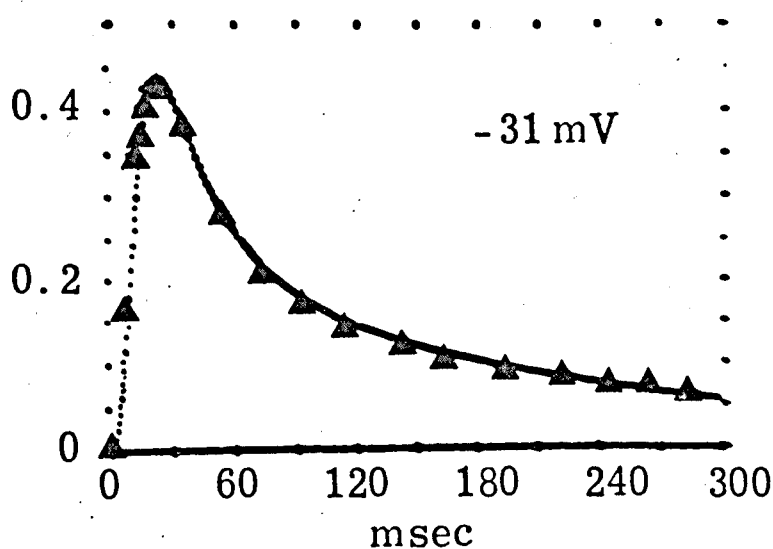
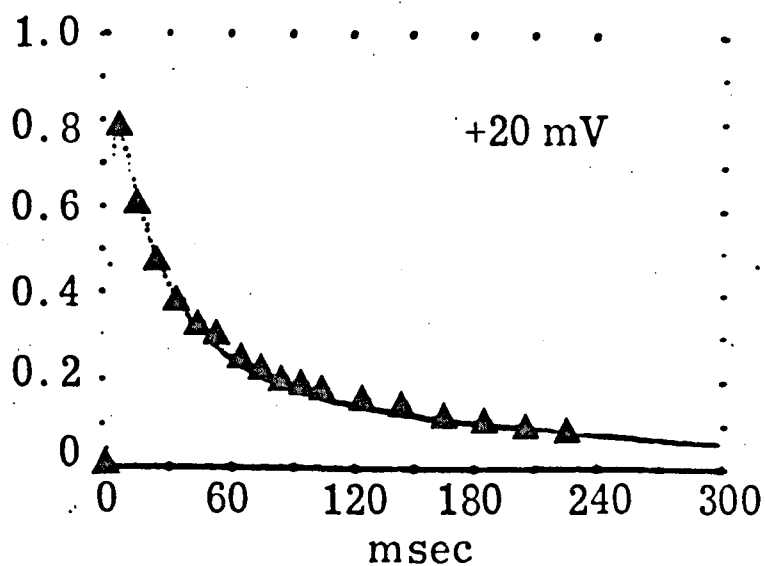


Fig. 37. Plots of equation III₃₁ for 3 values of T_b and T_n : Triangles show measured conductances from a fibre bathed in 1 mmole/l 4-AP (pH 7.2) and depolarized to the levels shown. Fibre F1 100276I.

III.iii The effect of 4-AP⁺ on the delayed Potassium Conductance

Blocking actions of 4-AP^+_o .

In section III_i, it was suggested that a major part of the reduction in peak current by 4-aminopyridine during a strong depolarizing step at pH 7.2 and 5.0 was due to the actions of the cationic form of 4-AP in the extracellular solution 4-AP^+_o . In the voltage range +10 mV to +80 mV the reduction in peak conductance by 4-AP 1mmole/l remains constant at approximately 50%; consequently in this voltage range the block induced by 4-AP^+_o may be regarded as independent of the membrane potential and the amplitude of the current flowing.

At more negative potentials it is difficult to determine the effect of 4-AP^+_o alone since at pH 7.2, the peak current may also be reduced by 4-AP^+_i , and to a greater extent at more negative potentials. In order to circumvent this problem, the effect of 4-AP^+_o on the instantaneous current voltage relation has been examined. In these experiments the blocking action of 4-AP^+_o can be examined before the channels become inactivated by 4-AP^+_i . An alternative method would be to do the experiments in acid solution, but there is the possibility that blocking actions of H^+ may also be voltage dependent, (see section III_{ix}) as it is in nerve (Woodhull, 1972).

The effect of 4-AP on the instantaneous current voltage curve

In a fully activated delayed rectifier the currents which flow 'instantaneously' after a change in the membrane potential are found to obey Ohm's Law (Adrian, Chandler & Hodgkin, 1970; Hutter, 1970, Argibay, 1974). Figures 44(a) & 44(b) show examples of current records which can be used to obtain the instantaneous current voltage relation in the absence and presence of 4-AP respectively. The potassium conductance is first fully activated and then a repolarizing step is made. The current flowing at the beginning of the repolarizing step was taken as the instantaneous current (I)_o at that voltage; it is assumed that there is no instantaneous change in the degree

of activation or inactivation at that time. Since the peak instantaneous current is often masked by the capacity transient, it was found necessary to obtain I_0 by extrapolation of semilogarithmic plots of the tail current to $t = 0$, as shown in Fig. 38.

In the absence of 4-AP (Fig. 38), the tail currents decay with an exponential time course described by the equation $I_t = I_0(\exp^{-t/T})$ from which T_n can easily be obtained.

In the presence of 4-AP (Fig. 39), it is found that the decay of tail current cannot be described by a single exponential time constant, but can be described by the sum of two exponentials such that the current at any time t is given by:

$$I_t = A \exp^{-t/T_a} + B \exp^{-t/T_b} \quad (*)$$

The instantaneous current is thus given at $t = 0$, i.e. by the sum $A + B$. Fig. 40 (open symbols) shows the instantaneous IV relation from untreated fibre. In the voltage range ± 90 mV the relationship is reasonably linear. The instantaneous IV relation for the same fibre in the presence of 1 mM 4-AP, calculated using equation (*) is also shown (filled symbols). In the voltage range -90 mV to $+90$ mV, the current voltage relationship remains linear but the membrane conductance is reduced by approximately 50%. In this case it is assumed that the rate constants T_a and T_b are attained instantaneously.

As a possible explanation for the two components in the decay of tail current in the presence of 4-AP it may be that fast component represents an additional voltage and time dependent block of the channels by 4-AP^+_o whilst the slow component represents channels which are not affected by 4-AP^+_o and are closing normally.

Voltage dependence of the rate of decay of tail current

Fig. 41 shows the voltage dependence of the rate of decay of the tail

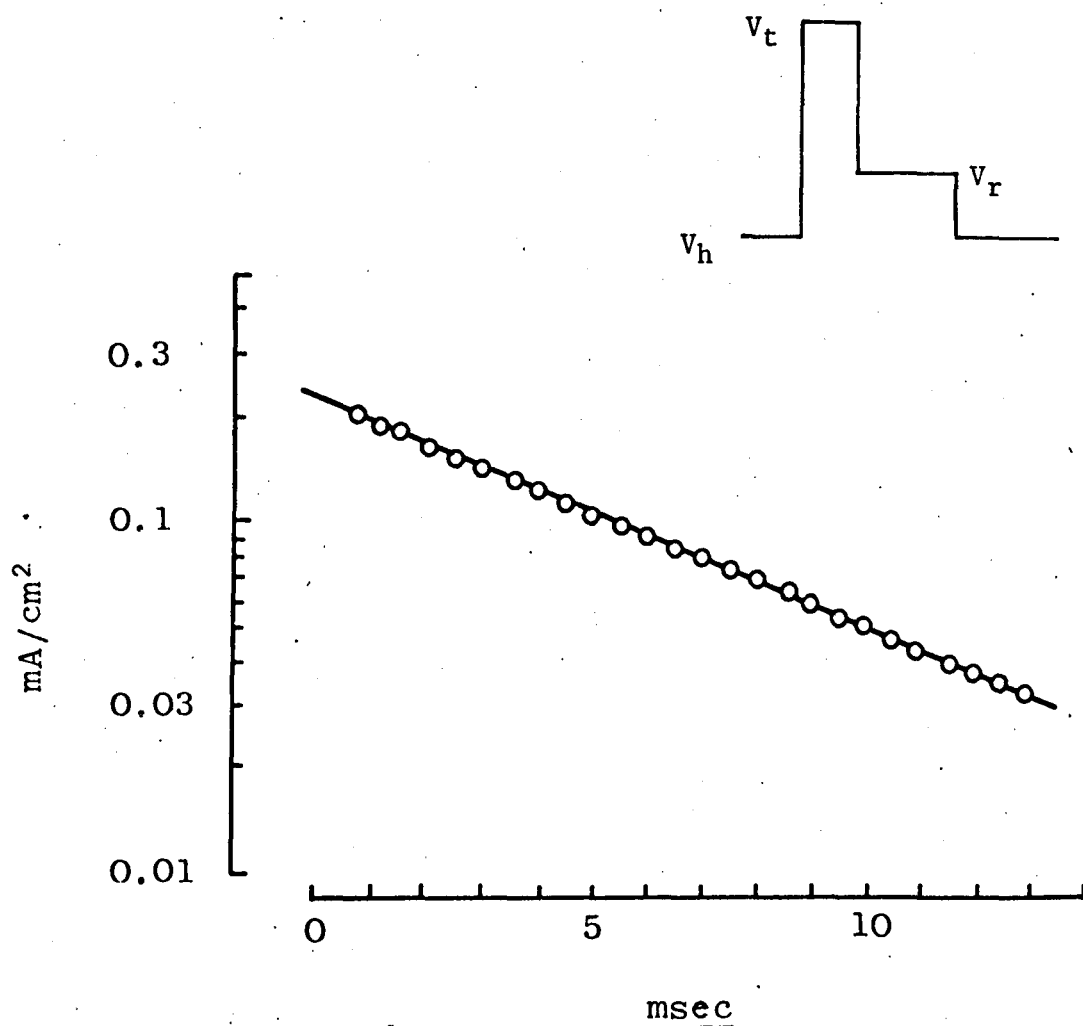


Fig. 38. Semi-logarithmic plot of the decay of inward tail current in the absence of 4-AP. Fibre F1 065751: $V_t = +30$ mV; $V_r = -70$ mV; $V_h = -122$ mV. $l=200\mu$; $l'=50\mu$; $a=70\mu$; pH 7.2; Temp. 21°C . Curve fitted by eye. Inset shows diagrammatically the voltage protocol.

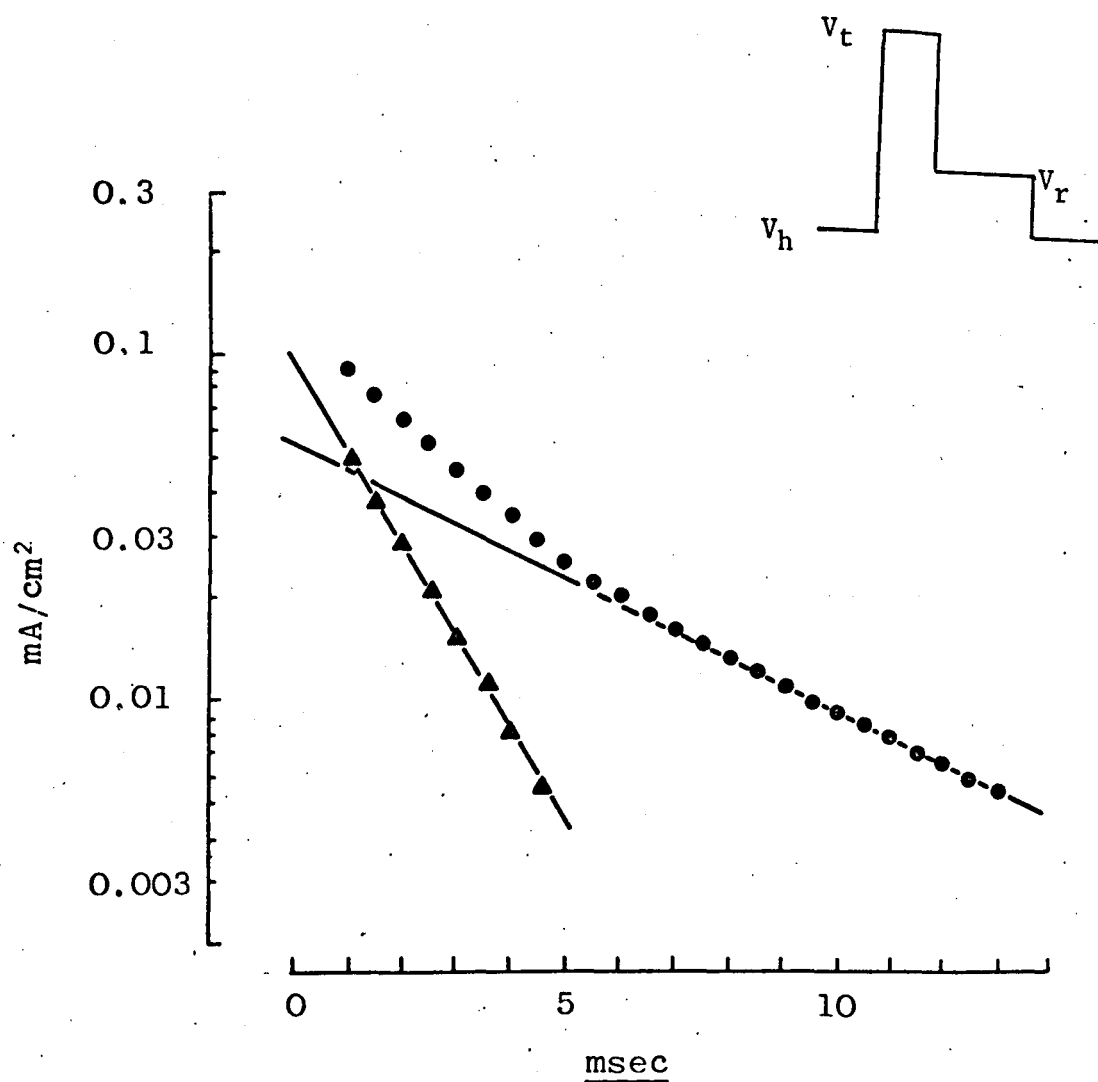


Fig. 39. Semi-logarithmic plot of the decay of inward tail current in the presence of 1 mmole/l 4-AP (pH 7.2).
 Fibre F1 06575 I: $V_h = -122$ mV; $V_t = +30$ mV; $V_r = -70$ mV;
 $l=200\mu$; $l'=50\mu$; $a=70\mu$; Temp. 21°C . Curves fitted by eye.
 Inset shows diagrammatically the voltage protocol.

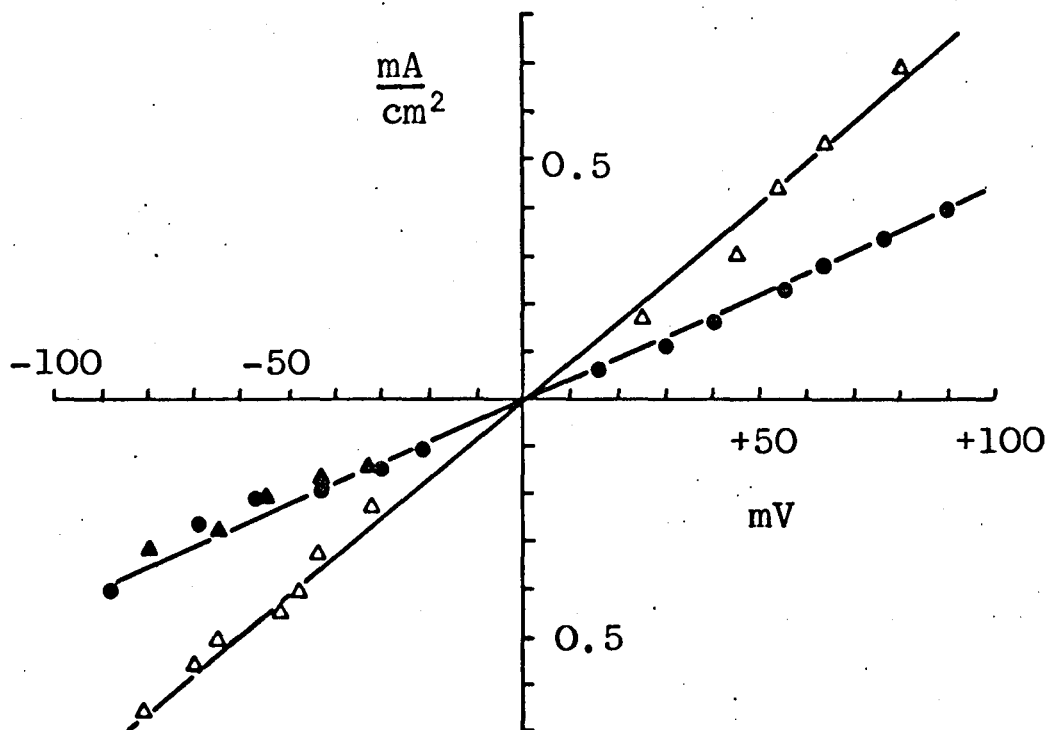


Fig. 40. Instantaneous current voltage relation for two fibres in isotonic KCH_3SO_4 solution before (Δ) and after 1 mmole/l 4-AP (pH 7.2) (\bullet). Temp. 20°C . Straight lines fitted by eye.

current in the absence of 4-AP (open symbols) and the rate of decay of the slower component in the presence of 4-AP. The rates shown are one quarter of the experimentally determined rates, this procedure takes account of the fact that the activation process is governed by n^4 kinetics. The slow component in the presence of 4-AP behaves in a similar manner to the untreated decay; however, at all voltages the rate constant of the slow component is larger than that of the untreated decay. This suggests that 'n' kinetics are slightly speeded by 4-AP. Such a situation would provide an explanation for the earlier observation of an increase in peak conductance seen with low concentrations of 4-AP. If the currents activate more quickly in the presence of low concentrations of 4-AP, then more channels will enter the conducting state before becoming inactivated, hence increasing the peak conductance.

The voltage dependence of the rate constant of the fast component is shown in Fig. 42. Despite the large scatter in the data, the results suggest that the rate of this process increases as the internal membrane potential is made more negative.

The Fraction of Channels Blocked by 4-AP^+_o .

The proportion of the available channels that become blocked by 4-AP in the course of a repolarizing pulse can be obtained from the ratio $A/A + B$. Fig. 43 shows the voltage dependence of $A + B$, the total instantaneous current and B the instantaneous current flowing through the unblocked channels. The fraction of channels blocked during repolarizing steps to -30 mV, -50 mV and -70 mV is 0.48, 0.64 and 0.7 respectively.

These results suggest the existence of a second voltage and time dependent block of the delayed potassium channel, in this case by 4-AP^+_o . As the internal potential is made more negative, both the rate and extent of this block increases. This suggests that 4-AP^+_o is pulled into the membrane upon repolarization. This is in contrast to the induced inactivation

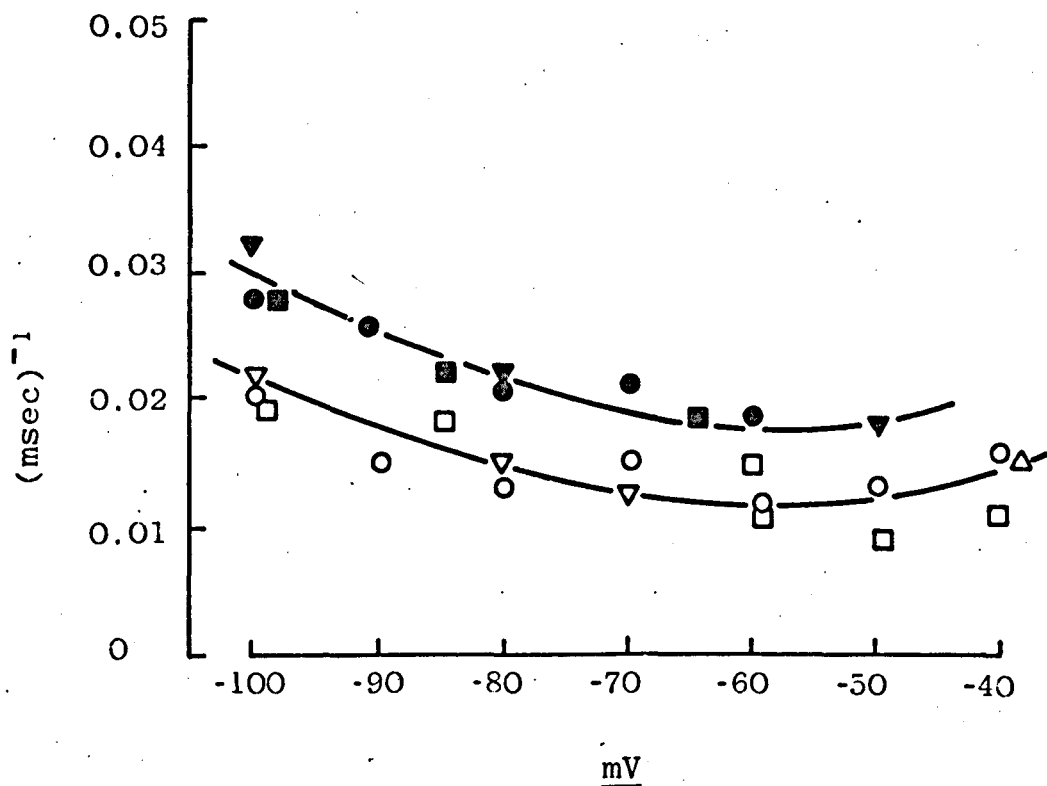


Fig. 41. The voltage dependence of the rate of decay of
 (a) the tail current in the absence of 4-AP
 (open symbols) and (b) the slow component of the decay of
 tail current in the presence of 1 mmole/l 4-AP. (pH 7.2),
 (filled symbols). Temp. 21°C.

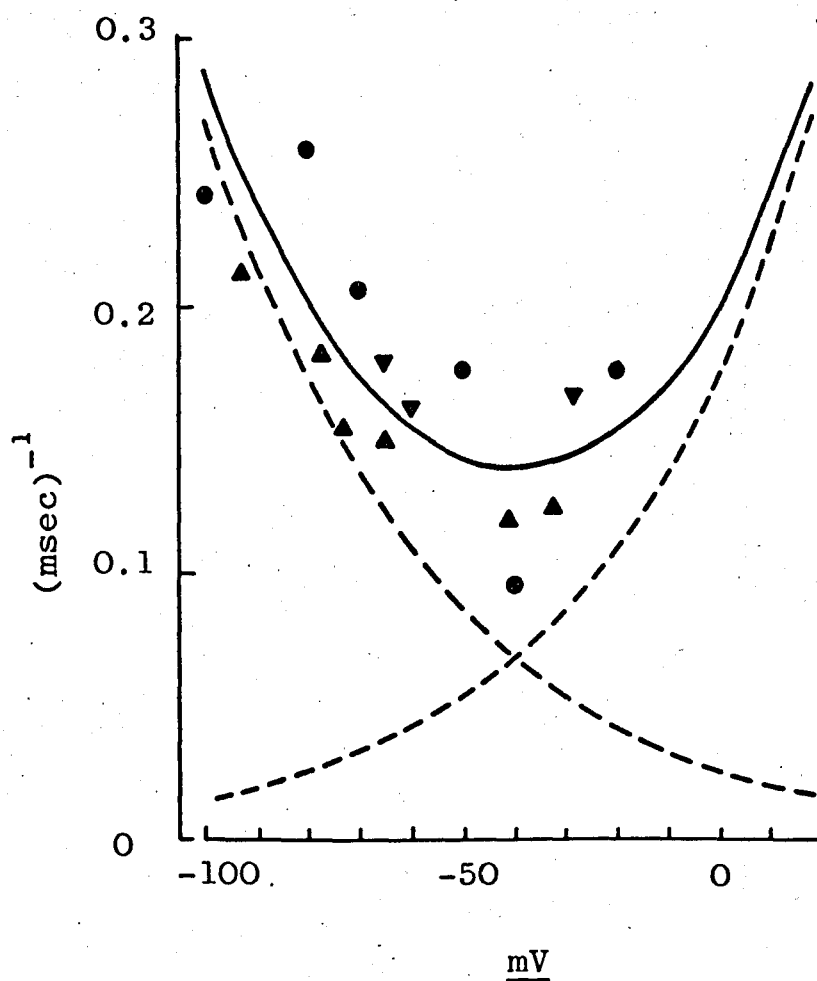


Fig. 42. The voltage dependence of the rate constant of the fast component of the inward tail current in the presence of 1 mmole/l 4-AP (pH 7.2). Dotted curves drawn according to equation III₃₇ and III₃₈ and the solid curve according to equation III₃₆. Temp. 21°C.

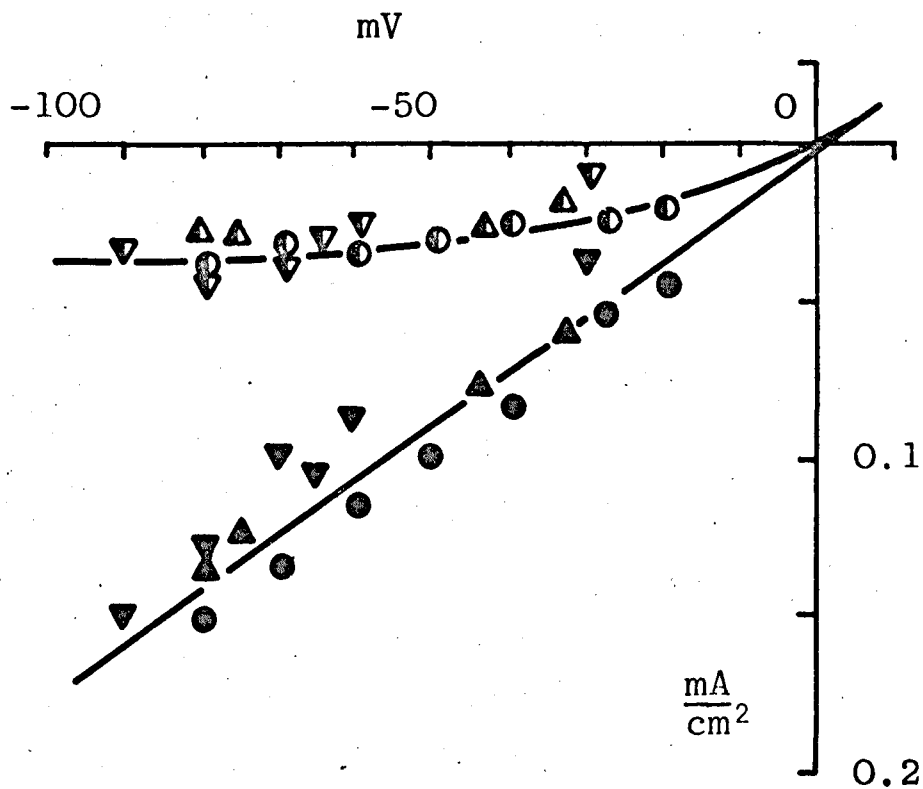


Fig. 43. Plot of the total instantaneous current in the presence of 1 mmole/l 4-AP (pH 7.2), (filled symbols); and the instantaneous current flowing through channels that are not blocked by 4-AP, (half filled symbols). Temp. 21°C. Curves fitted by eye.

by 4-AP_i^+ described in Section III_{ii}, which increases in rate and extent as the internal potential is made more positive.

If the membrane potential is maintained highly negative after an early repolarizing step then the induced block persists and results in a reduction in the maximum available peak conductance determined by a subsequent large depolarizing step. The fraction of channels blocked can then be obtained from the ratio of the peak conductance before and after the conditioning pulse.

Fig. 44(a) shows the voltage protocol used in such an experiment and (b) shows examples of the current records obtained. From the holding potential (V_H) the membrane is fully activated by a strong brief depolarizing step (V_t)₁; the membrane is then repolarized to a ^{conditioning} level (V_c) and from there, after several seconds, to the holding potential (V_H). After an interval of ~ 10 sec, to allow adequate repriming of the delayed rectifier, the effect of the early repolarizing step to V_c on the maximum available peak conductance is determined by imposing a second strong depolarizing pulse (V_t)₂. In practice (V_t)₂ was followed by an early repolarizing step and its effect examined by a subsequent test step (V_t)₃.

In the current records shown, the value of the conditioning voltage that preceeds each current record is shown below that trace. When the conditioning voltage is 0 mV the peak potassium conductance is approximately 50% of the untreated value. After a conditioning step to -80 mV, the peak conductance is reduced to approximately 15% of the untreated value. A second and third conditioning pulse to -80 mV produces a further small decrease in the peak conductance. This reduction in peak conductance can be reversed if the conditioning potential is returned to 0 mV. In the absence of 4-AP this procedure of early repolarization has no effect on the peak conductance as can be seen in Fig. 44(c).

The extent of the reduction in the peak conductance seen after early repolarization in the presence of 4-AP is found to depend on the value of V_c .

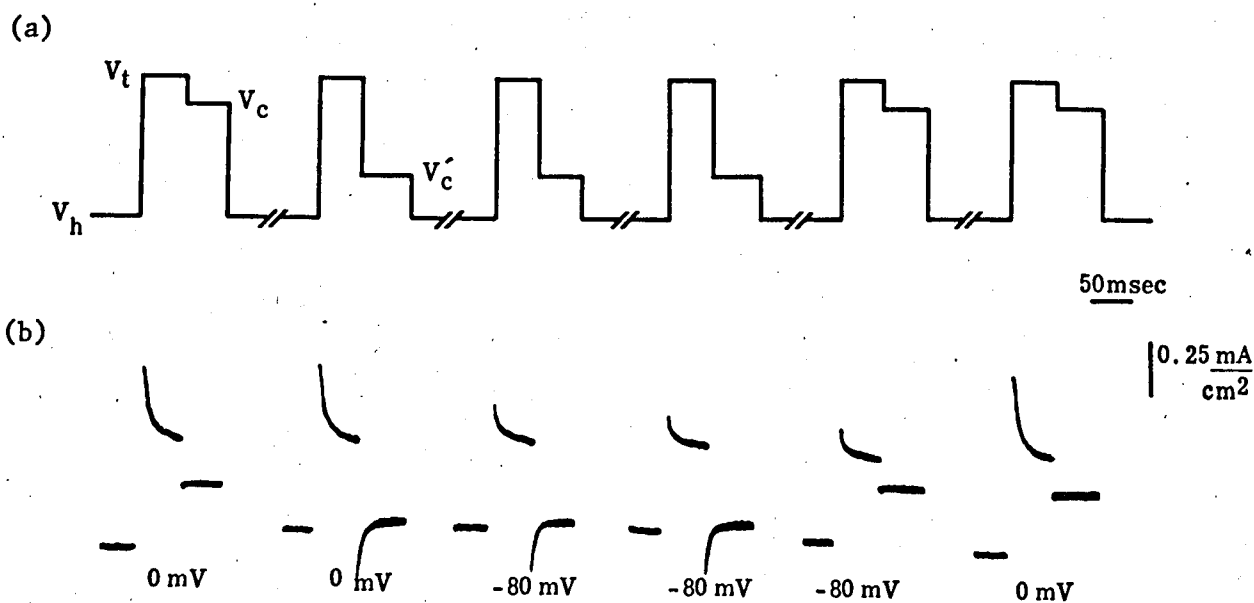


Fig. 44. The effect of early repolarization on the peak potassium current in the presence of 1 mmole/l 4-AP (pH 7.2). Upper section shows diagrammatically the voltage protocol. Below is shown records of the membrane current. The figures below each current record show the value of the conditioning step prior to that test pulse. Fibre F1 150975I; $V_h = -122$ mV; $V_t = +34$ mV; $V_c = 0$ mV; $V'_c = -80$ mV; $l = 200 \mu$; $l' = 80 \mu$; $a = 80 \mu$; Temp. 21° C.

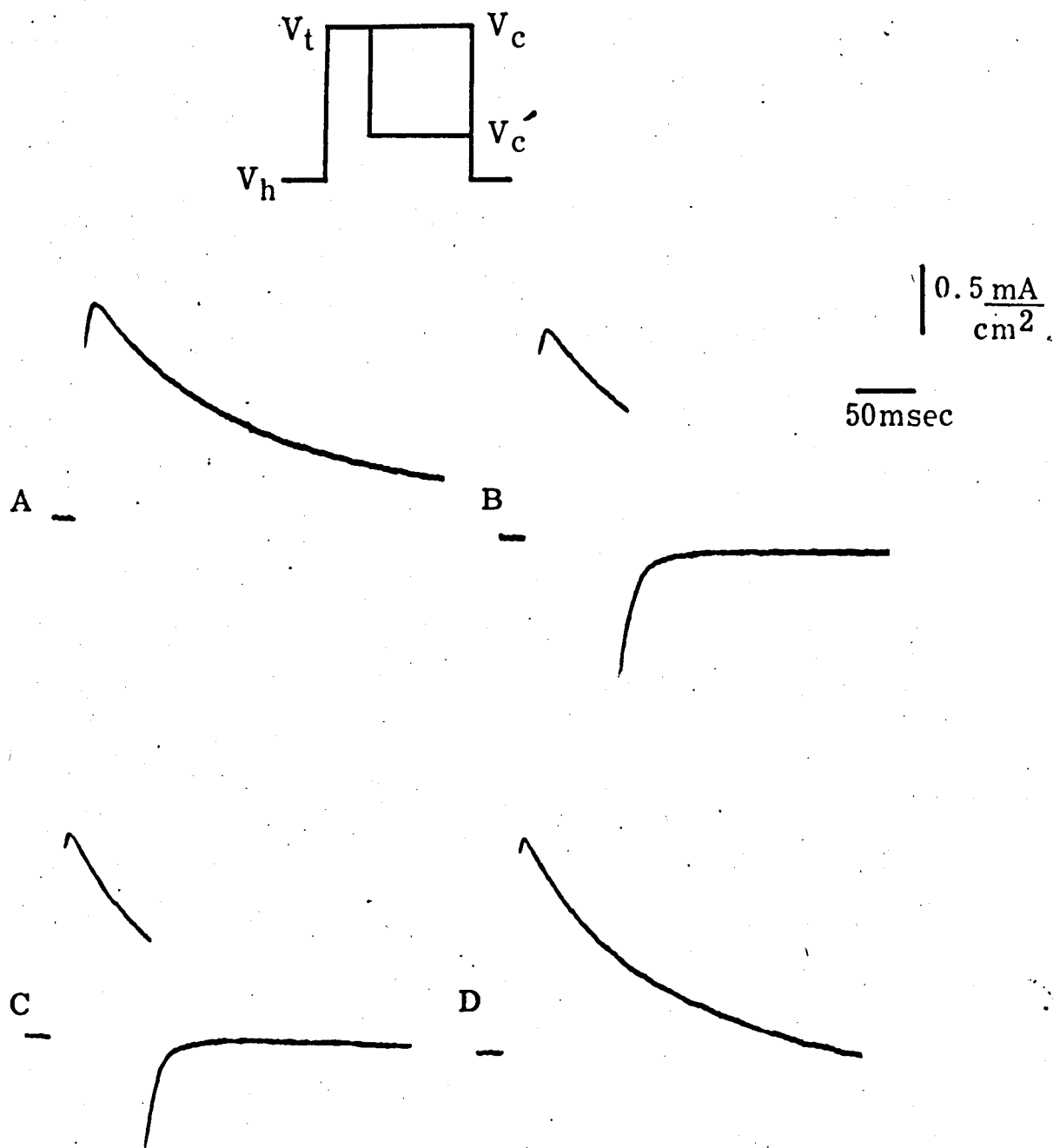


Fig. 44 (c). The effect of early repolarization on the peak potassium current in the absence of 4-AP. Inset shows diagrammatically the voltage protocol used. $V_h = -122 \text{ mV}$; $V_t = +29 \text{ mV}$; $V_c = +29 \text{ mV}$; $V'_c = -106 \text{ mV}$. Fibre F1 131076 $l = 200 \mu$; $l' = 50 \mu$; $a = 80 \mu$; Temp. 20°C .

As the conditioning potential is made negative the block is found to increase; when V_c is made positive ($> + 30$ mV) there is little or no alteration in the degree of block and the peak conductance remains approximately 50% of the untreated value. Fig. 45 shows the fraction of channels that become blocked in the course of a repolarizing step as determined from the decay of tail current (open symbols); and also the reduction in peak conductance after a maintained early repolarization (filled symbols). The data is obtained from 15 different fibres and consequently there is a degree scatter in the points. As can be seen the results from each type of experiment lie on the same curve.

The blocking action of 4-AP_o^+ , can then be divided into two components. In keeping with other workers (see page 88) these are called the "tonic" component which under these conditions is responsible for the 50% reduction in conductance at positive potentials; and the "phasic" component which intensifies the block at more negative potentials.

It is important to determine whether the phasic block results from an effect of potential on the position of 4-AP_o^+ ion or whether the block is caused by 4-AP_o^+ being swept into the channel as the result of inward current flow during the repolarizing step. There are two pieces of evidence that suggest the 'phasic' block is dependent upon the membrane potential and not membrane current. Fig. 46 shows the results from four fibres in which the repolarizing step was made at 50 msec, 150 msec, 250 msec, and 500 msec after the initial depolarization. In each case, when \bar{g}_K is determined upon subsequent depolarization it is found to be reduced, despite the fact that the inward current tail flowing during the repolarizing step becomes smaller as the duration of the depolarizing steps increases. In section (d) inward tail is negligible due to normal and 4-AP_i^+ induced inactivation. Since the phasic block proceeds independently of the magnitude of the current tail this argues against a current dependent block.

Fig. 45 & 45' shows the pulse dependent block and its partial reversal. After early repolarization there is the subsequent reduction in g_K ; upon repolarization to zero mV the block is partially reversed as determined during the next depolarizing step. In each case this reversal could only have taken place during the previous voltage step to zero mV, at which time no current was flowing through the channel. This also tends to support the suggestion that the phasic block is a voltage dependent reaction.

The phasic block is apparent after a depolarization which lasts between 50 msec and 500 msec, Fig. 46, but however, is not present if repolarization is delayed for several seconds. As can be seen in Fig. 18 (pH 7.2) the peak conductance of fibres depolarized for 3 sec and then repolarized to -122 mV is only reduced by 50%; the level of the tonic block alone. In order to account for such a discrepancy it may be supposed that upon prolonged depolarization a conformational change occurs near the outer surface of the channel such that upon repolarization 4-AP^+_o is denied access to its blocking site. This conformational change cannot be directly related to the inactivation mechanism which causes the exponential decay of current since the 4-AP^+_o block can still be induced in channels that are in different states of inactivation (see Fig. 46). This suggests that the conformational change occurs more slowly than normal inactivation. It may therefore be associated with the processes of deepening inactivation as described by Argibay ^{or Hutter} (1973).

The mechanism of block by 4-AP^+_o .

The main features of the channel blockage by 4-AP^+_o are: (a) a voltage independent or 'tonic' component; (b) a voltage dependent block which develops rapidly and increases as the internal potential is made more negative; (c) the block is not apparently affected by 'n' kinetics but may be influenced by a process associated with inactivation of the channel.

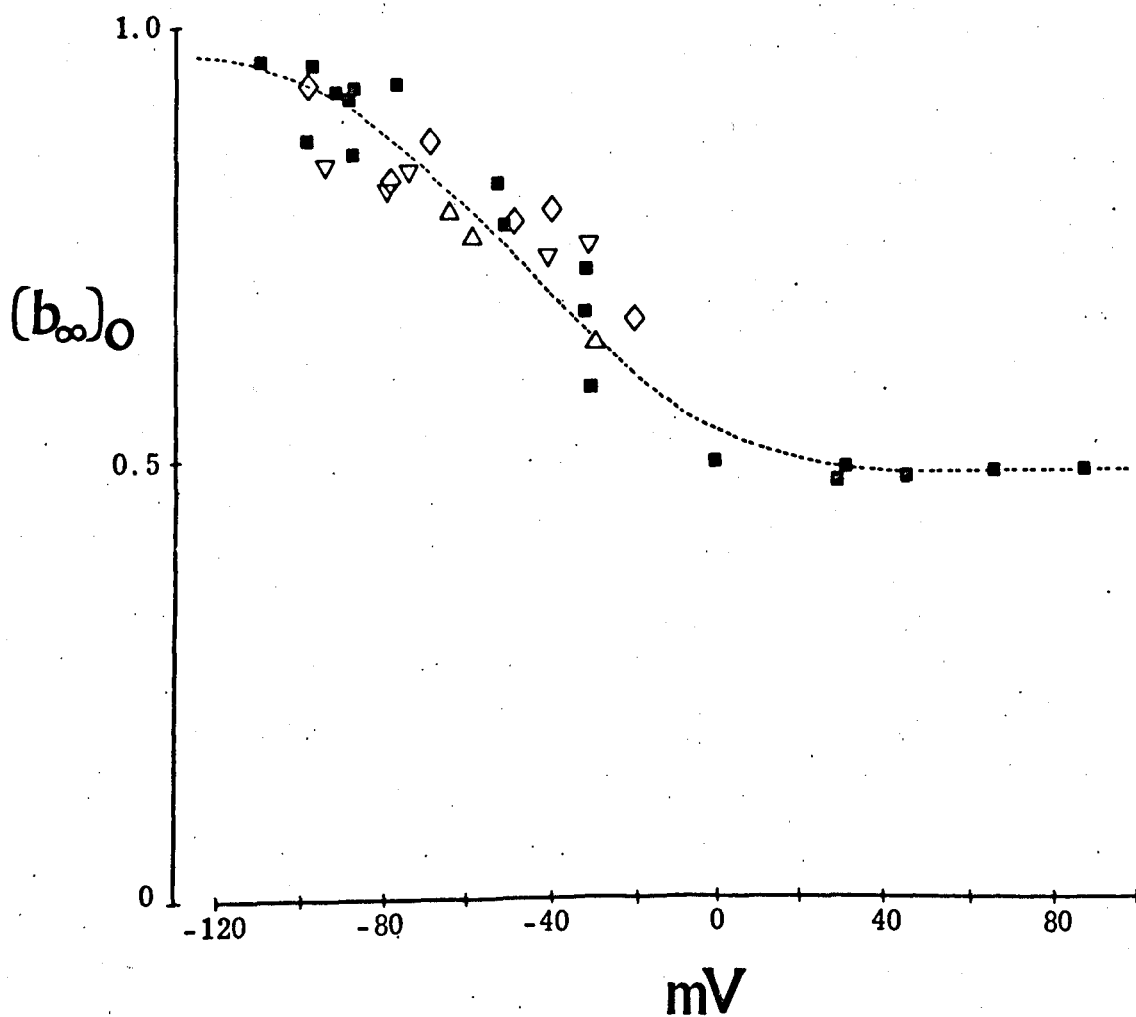


Fig. 45. The voltage dependence fraction of channels blocked by 4-AP_0^+ . Data from (a) the decay of inward tail current (open symbols); and (b) the effect of early repolarization on the peak membrane conductance, (filled symbols). Temp. $19\text{-}22^\circ\text{C}$. Curve drawn according to equation III₃₄.

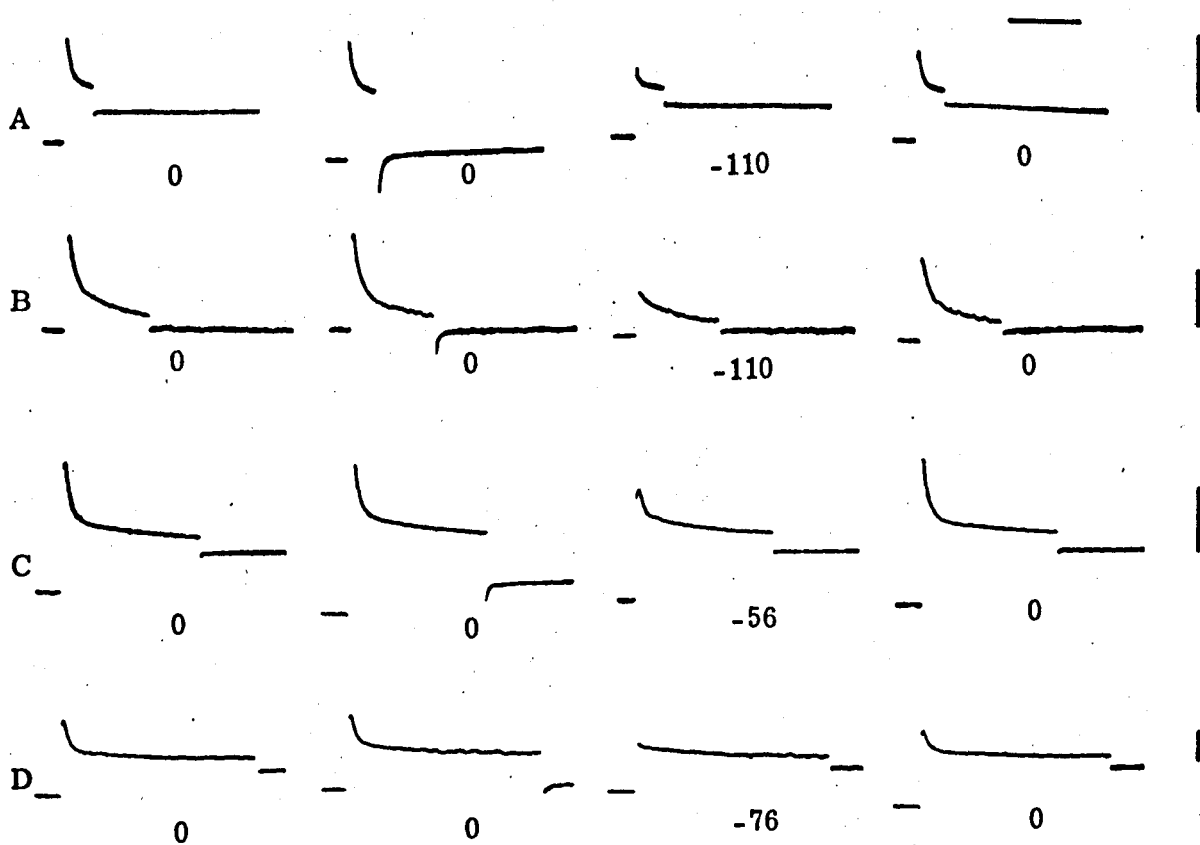
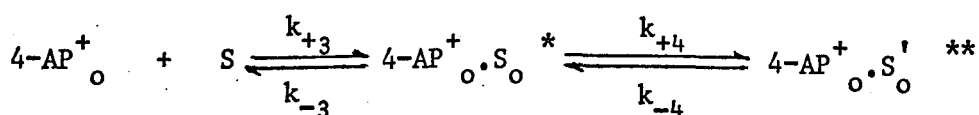


Fig. 45. The effect of repolarization of the membrane at different times on the peak potassium conductance in the presence of 1 mmole/l 4-AP. Records from 4 different fibres; values for the conditioning voltages show below each current record. Record A: Fibre F1 290976 $V_h = -122\text{mV}$; $V_t = +32\text{mV}$; $V_c' = -110\text{mV}$; $l = 250\mu$; $l' = 80\mu$; $a = 80\mu$; Temp. 20°C . B: Fibre F4 010976 $V_h = -122\text{mV}$; $V_t = +22\text{mV}$; $V_c' = -110\text{mV}$; $l = 200\mu$; $l' = 50\mu$; $a = 80\mu$; Temp. 21°C . C: Fibre F1 141076II; $V_h = -122\text{mV}$; $V_t = +32\text{mV}$; $V_c' = -56\text{mV}$; $l = 200\mu$; $l' = 50\mu$; $a = 80\mu$; Temp. 19°C . D: Fibre F2 150975I; $V_h = -122\text{mV}$; $V_t = +34\text{mV}$; $V_c' = -76\text{mV}$; $l = 250\mu$; $l' = 50\mu$; $a = 100\mu$; Temp. 20°C . Vertical calibration bars 0.2 mA/cm^2 . Horizontal calibration bar 100 msec.

This situation can be described by two reactions involving 4-AP^+_o at two blocking sites, S_o on the outer surface of the membrane and S'_o lying within the channel. The dissociation constant of the reaction of S_o is independent of potential since it lies outside the electric field, whilst at S'_o the dissociation constant is voltage dependent. Such a scheme may be represented in the following way:-



where k_{+3} , k_{-3} , k_{+4} and k_{-4} are rate constants; k_{+4} and k_{-4} are voltage dependent; $4\text{-AP}^+_o \cdot S_o^*$ and $4\text{-AP}^+_o \cdot S'_o^{**}$ are channels blocked by 4-AP^+_o . This scheme is similar to that derived by Strichartz (1973) to account for the blocking actions of internally present lidocaine derivative, QX 314 on the sodium channel of frog node. The expression for the fraction of channels blocked in the steady state $(b_\infty)_o$ for this scheme is

$$(b_\infty)_o = \frac{1}{1 + 4\text{-AP} \left[\frac{k_{-3}}{k_{+3}} + \frac{k_{-3}}{k_{+3}} \cdot \frac{k_{-4}}{k_{+4}} \right]} \quad 111.34$$

As has already been discussed in Section III_{ii}, the expression for a voltage dependent dissociation constant may be given by

$$\frac{k_{-4}}{k_{+4}} = K^o \exp - \left(\frac{z\delta_4 EF}{RT} \right) \quad 111.35$$

where K^o is the dissociation constant in the absence of any electric field; z is the valency of the reactant; δ_4 is the fraction of the applied electric field, E , experienced by the reaction site; and RT and F have their usual meanings. At 20°C $\frac{RT}{F}$ is approximately 25 mV, and with 4-AP $z = +1$. Thus combining equations 111.34 and 111.35 the fraction of channels blocked by

4-AP^+_o is given by

$$(b_\infty)_o = \frac{1}{1 + 4\text{-AP}^+_o \left[\frac{k_{-3}}{k_{+3}} + \frac{k_{-3}}{k_{+3}} K^o \exp - \left(\frac{\delta_4 E}{25} \right) \right]} \quad 111.36$$

The experimental results shown in Fig. 45 can be fitted to equation III.36 with $\frac{k_{-3}}{k_{+3}} = 1.1 \text{ mM}$; $K^o = 0.08 \text{ mM}$; and $\delta' = 0.67$. δ' is the experimentally determined voltage dependence. If the field within the membrane is linear and there is a single site, a value of δ' of 0.67 suggests that the blocking site is located approximately 2/3 of the way across the membrane from the outside. However, as was noted in Section III_{ii} if there is more than one binding site for the blocking ion or any other permeant ion within the electric field and close to the blocking site the experimentally observed δ , $(\delta)'$, may be the sum of the δ 's for each site. The voltage independent block induced by 4-AP^+_o may be at such an adjacent site, thus $\delta' = (\delta_3 + \delta_4)$, where δ_3 is the fraction of the applied field at the site S_o . The tonic block however is not affected by potential, hence $\delta_3 = 0$; consequently the experimentally observed δ' equals $(0 + \delta_4) = \delta_4$. It is then likely that the blocking site S'_o , is located 2/3 of the way across the membrane.

The voltage dependence of the individual rate constants k_{+4} and k_{-4} can be calculated from the relation between the fraction of channels blocked and the potential:

$$(b_\infty)_o = \frac{k_{+4}}{k_{+4} + k_{-4}} \quad 111.37$$

and the voltage dependence of the rate of the block:

$$(T_b)_o^{-1} = k_{+4} + k_{-4} \quad 111.38$$

From the data available:

$$k_{+4} = 0.07 \exp \frac{(V - 40) \text{ mV}}{38} \text{ msec}^{-1} \quad 111.39$$

$$k_{-4} = 0.07 \exp\left(-\frac{(V - 40)}{38}\right) \text{msec}^{-1}$$

111.40

The results of the blocking actions of 4-AP^+_o have been analysed in terms of two independent blocking sites. An alternative explanation might be that there are two populations of channels. One in which the blocking site is on the outer surface of the membrane outside the electric field; and a second population with a blocking site within the channel. However, two populations of potassium channels seems unlikely for the following reason. The dose response relationship of 4-AP at pH 5.0 (Fig. 20) describes the blocking action of 4-AP outside the cell. Since all these experiments were done with long, strong depolarizing steps they describe only the tonic component of the 4-AP^+_o block. With high concentrations the majority of the channels become blocked. This suggests that all channels have a "tonic" blocking site. Under the appropriate conditions phasic block can be induced in unblocked channels. This argues that some channels, have two blocking sites and it seems likely then that all channels have two blocking sites for 4-AP^+_o .

In the previous section which analysed the blocking actions of 4-AP^+_i it was tacitly assumed that the number of available channels was reduced by 4-AP^+_o to the same extent at all membrane potentials. In this section however, it has been shown that there is a component of 4-AP^+_o block which can be influenced by the membrane electric field. It is necessary therefore to consider whether the simplifying assumption used in the previous section is now valid.

All of the experiments that analyse the time course of 4-AP^+_i induced inactivation were done on fibres that were depolarized for at least 3 sec before they were returned to the holding potential for repriming. Under these conditions only the tonic component of the 4-AP^+_o block is present. Consequently the maximum available conductance is reduced by only the tonic component and the block induced by 4-AP^+_i . The analysis of the induced

inactivation with the assumption of a constant number of potentially available channels is therefore probably valid.

It is also important to consider whether the 4-AP^+_i induced block will affect the analysis of the 4-AP^+_o block. In the pulsing experiments the effect of 4-AP^+_o was assessed by a reduction on the peak conductance determined at the beginning of a strong depolarizing step. Since the opening of the channels is rapid in this situation and the rate of induced inactivation relatively slow, the peak conductance is not reduced by 4-AP^+_i and is a reliable measure of the block due to 4-AP^+_o . Changes in the magnitude of the peak current under these conditions therefore only represent changes in the level of 4-AP^+_o block.

III. iv. The effect of 4-AP on the Action Potential

The Effects of 4-AP on the Action Potential

The effects of 4-AP on the action potential have been examined. The mechanical response which inevitably follows an action potential was reduced in these experiments by bathing the muscle in Ringer solution^{solution A.} made hypertonic with 500 mM sucrose. Fibres were stimulated via an intracellular micro-electrode impaled several mm away. Under these conditions many action potentials could be recorded in the same fibre without causing any serious damage.

Fig. 46 shows the effect of 2 mmole/l 4-AP (pH 7.2) on the intracellularly recorded action potential. The control potential, in the absence of 4-AP is shown on the left, and the remaining records were recorded at 30 sec intervals after the addition of 4-AP to the bath. With progressively longer exposure there is a marked slowing of the repolarizing phase. The duration of the action potential, as determined as the duration at half maximum amplitude, was prolonged from 1.6 msec to 3.3 msec. The overshoot and the rate of rise of the action potential is scarcely affected as is the resting potential. Such a result was to be expected in view of the effect of 4-AP on the maximum potassium conductance.

Use Dependent Prolongation of the Action Potential

An unexpected finding was that the prolongation of the action potential by 4-AP seemed to be dependent upon how often the fibre was stimulated.

Fig. 47 shows records from 4 fibres from one muscle which demonstrates this use dependent phenomenon. Section A shows action potentials recorded in the absence of 4-AP at a frequency of 0.1 Hz. Each action potential is assigned a number which denotes the number of previous impulses in that fibre. In the absence of 4-AP there is no effect of activity on the duration of the action potential at this frequency. Section B shows action potentials recorded in the same fibre as in A approximately 10 min after the addition of 4-AP;

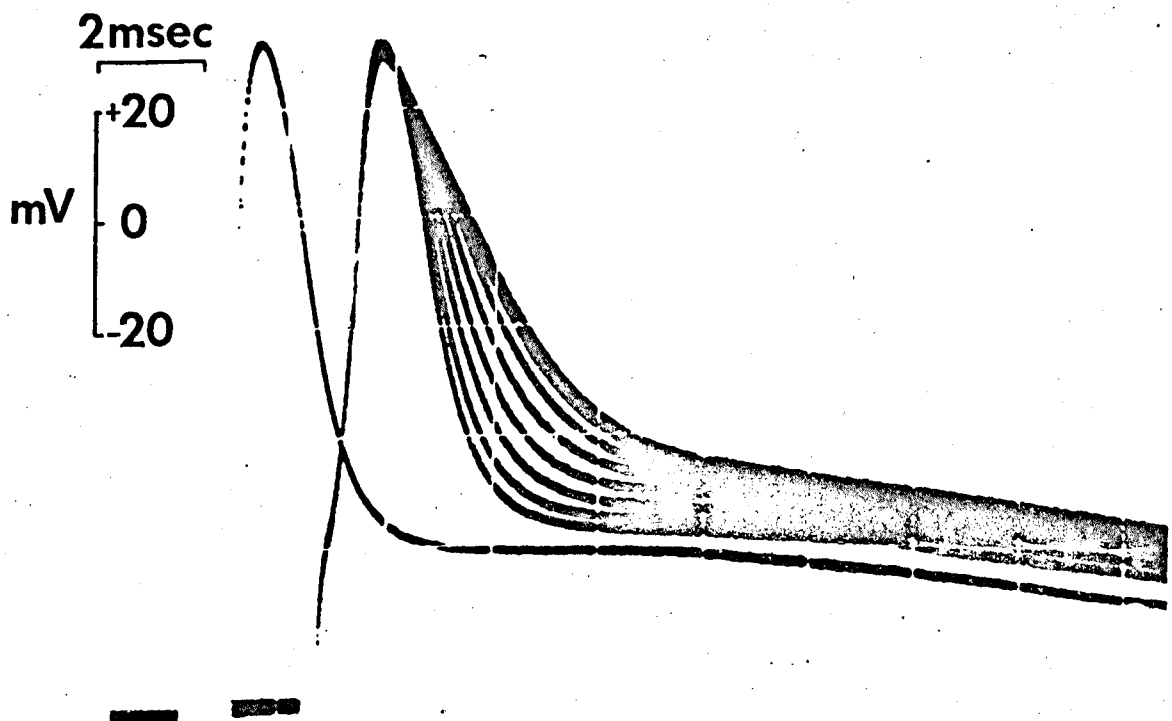


Fig. 46. The effect of 2 mmole/l 4-AP (pH 7.2) on the muscle action potential. Control record on the left; the remainder after the addition of 4-AP to the bath. Stimulating frequency 1 per 30 sec. Fibre 08175 II; Temp. 21°C.

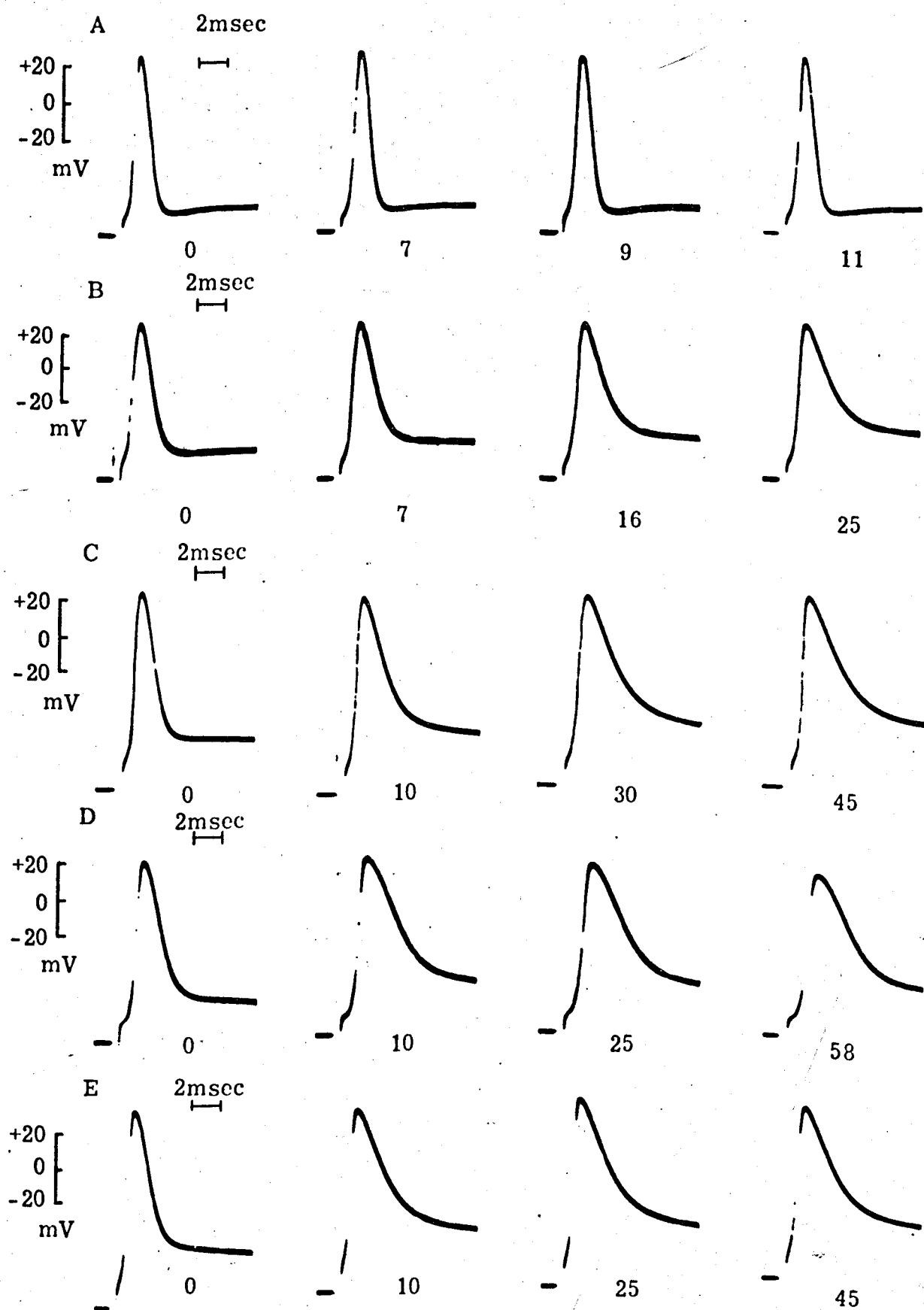


Fig. 47.

Fig. 47. The effect of 1 mmole/l 4-AP on the muscle action potential. Row A & B: action potentials recorded in the same fibre (A) before and (B) 10 min after the addition of 4-AP to the bathing solution. Fibre unstimulated during the equilibration period. Rows C,D and E, action potential recorded from different fibres in the same muscle, impaled at 20, 35 and 50 min respectively after the addition of 4-AP. Numbers below each record represent the number of previous stimuli in that fibre in that solution. $pH_o = 7.2$; stimulating frequency 0.1 Hz in all fibres; Temp. $22^{\circ}C$. Experiment 081751.

during the equilibration period the fibre was left unstimulated. Upon stimulation the action potential gets progressively longer. This result is similar to that described above. After 24 min exposure to 4-AP a second fibre was impaled, the action potentials recorded in this fibre are shown in Section C. The duration of the first action potential in this fibre ($N^0 = 0$) was only slightly longer than that of the untreated fibre and much less than the last action potential in the previous fibre. As the number of previous stimuli is increased the action potential becomes progressively longer. Sections D & E show records from two other fibres in the same muscle that were exposed to 4-AP for 35 and 50 min prior to being stimulated. In each case the duration of the first action potential is not as prolonged as might be expected from the duration of the final action potential in the previous fibre. Upon stimulation the action potential grew progressively longer. This use dependent prolongation was observed in all polarized fibres that were left unstimulated in the presence of 4-AP.

It is interesting to note that the increment of increase in duration per impulse increases for fibres exposed to 4-AP for longer times. This suggests that at these late times the fraction of channels blocked per impulse becomes larger.

The Use Dependent Prolongation at Different Extracellular pH

Fig. 48 shows the results from three experiments which examine the effect of 2 mmole/l 4-AP on the duration of the action potential at different extracellular pH. Stimulation in each fibre was begun approximately 10 min after the addition of 4-AP to the bath. The use dependent prolongation of the action potential is present at pH 7.2 and 9.8 but apparently not in acid solution. The increase in duration per impulse is much greater in alkaline solution. A possible interpretation of these results is that 4-AP, present internally, is responsible for the prolongation. The dependence of the block on the fibre being stimulated may be a reflection of the finding in the voltage clamp experiments that the block by 4-AP^+_i can only proceed in

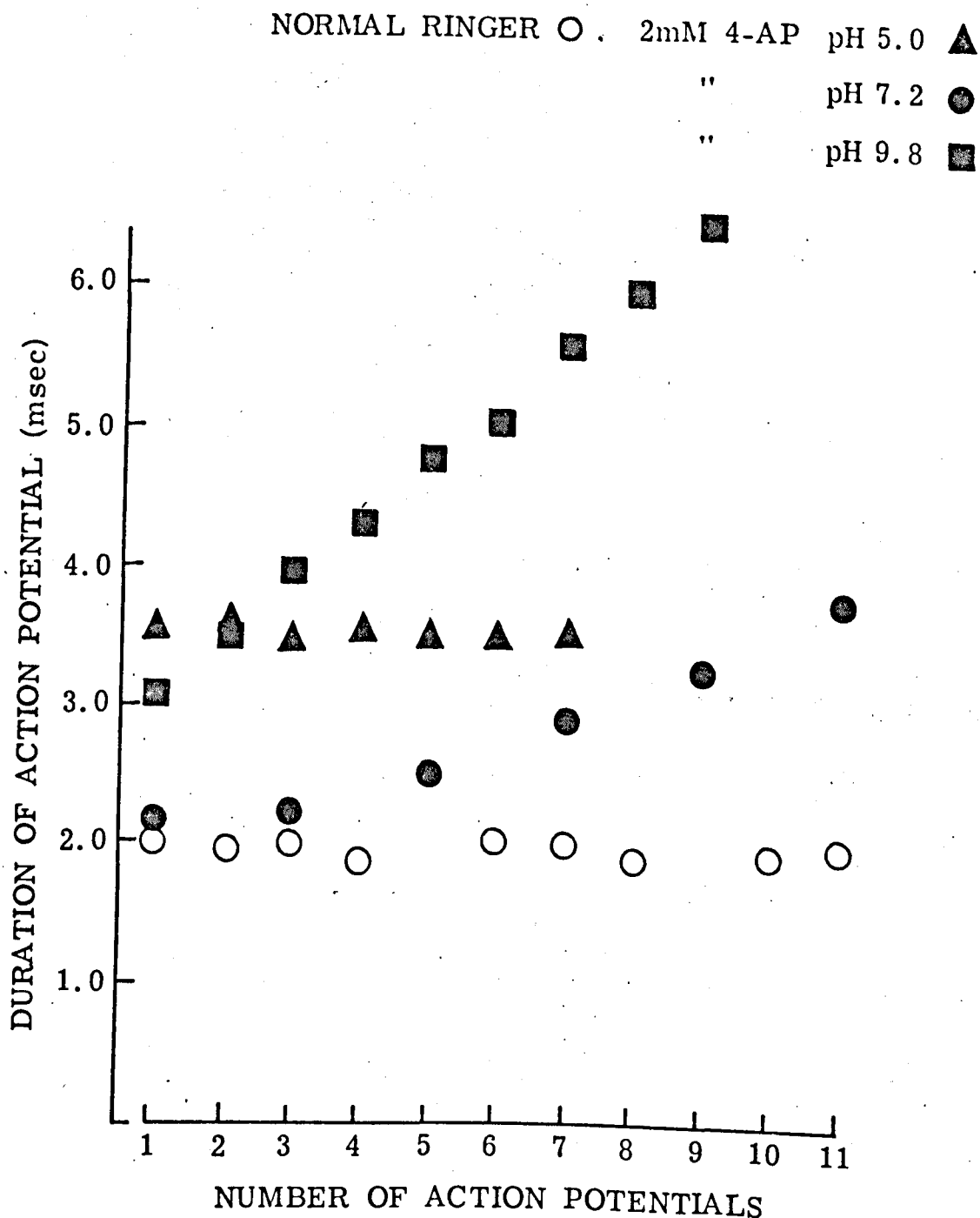


Fig. 48. The effect of 2 mmole/l 4-AP on the duration of the action potential recorded in solution at different extracellular pH. Open circles, untreated fibre pH 7.2; filled circles, same fibre 10 min after the addition of 4-AP to bathing solution; filled squares, the duration of action potentials recorded in a fibre 10 min after the addition of 4-AP at extracellular pH 9.8; filled triangles, duration of action potentials recorded 10 min after the addition of 4-AP at extracellular pH 5.0.

open channels. In the polarized membrane the activation gates are closed, and may deny 4-AP access to the blocking site. Upon stimulation these gates open transiently and a proportion of the channels become blocked, thereby prolonging the next action potential.

In interpreting these results at different pH it must be borne in mind that Cl^- ions play an important role in the repolarizing phase of the action potential (Hutter & Noble, 1960) and that the chloride conductance is modified by the extracellular pH (Hutter & Warner, 1967; Warner, 1972). The instantaneous chloride conductance is highest in alkaline solution and lowest in acid; consequently in acid solution the action potentials are already prolonged. If the above interpretation of the use dependent prolongation were correct then the phenomenon should be absent in acid solution. This would appear to be the case but caution must be taken in view of the effects of pH per se on the duration of the action potential.

III. v. The effect of 4-AP on the Inward Rectifier

The Effect of 4-AP on the Inward Rectifier

In fibres bathed in isotonic KCH_3SO_4 solution the potassium equilibrium potential and the resting potential are close to zero. Under these conditions, in the absence of formaldehyde the membrane current is carried by potassium ions through the inward rectifier and leak channel. Fig. 49(b) shows records of the membrane current in such a fibre when it is polarized to -30, -80, +30 and +80 mV and Fig. 50 shows a plot of this data. In order to maintain the highly negative potential a large inward current is necessary whilst only a small current is required to maintain a potential of +80 mV. The membrane is thus rectifying in the inward direction. During the voltage step to -80 mV there is a partial repriming of the delayed rectifier and this is seen as a transient outward current upon depolarization to +30 mV. 5 mmole/l 4-AP (pH 7.2) was then added to the bath and its action on the membrane examined. The membrane currents after a 35 min soak in 4-AP solution are shown in Fig. 49(b) and Fig. 50. Inward rectification is reduced by approximately 20% at -80 mV. The delayed current originally seen on stepping the membrane to +30 mV is absent. This provides some additional indirect evidence that 4-AP blocks non formaldehyde treated delayed currents.

When the extracellular pH is changed from 7.2 to 9.8 the potency of 5 mM 4-AP in blocking the inwardly rectifying current increases. After only 8 min exposure in alkaline solution the inward current is reduced by approximately 80% at -80 mV. In fibres exposed to 5 mM 4-AP for longer times anomalous rectification could be completely abolished. From these results it would appear that the inward rectifier is more effectively blocked by 4-AP^+_i than 4-AP^+_o .

Stanfield (1970a) has shown that TEA ions in the extracellular solution (80 mM) reduces the inwardly rectifying currents by 80%. The reason for the difference in sensitivity of the channel for TEA and 4-AP^+_o is unclear; it is possible that high concentrations of 4-AP^+_o will

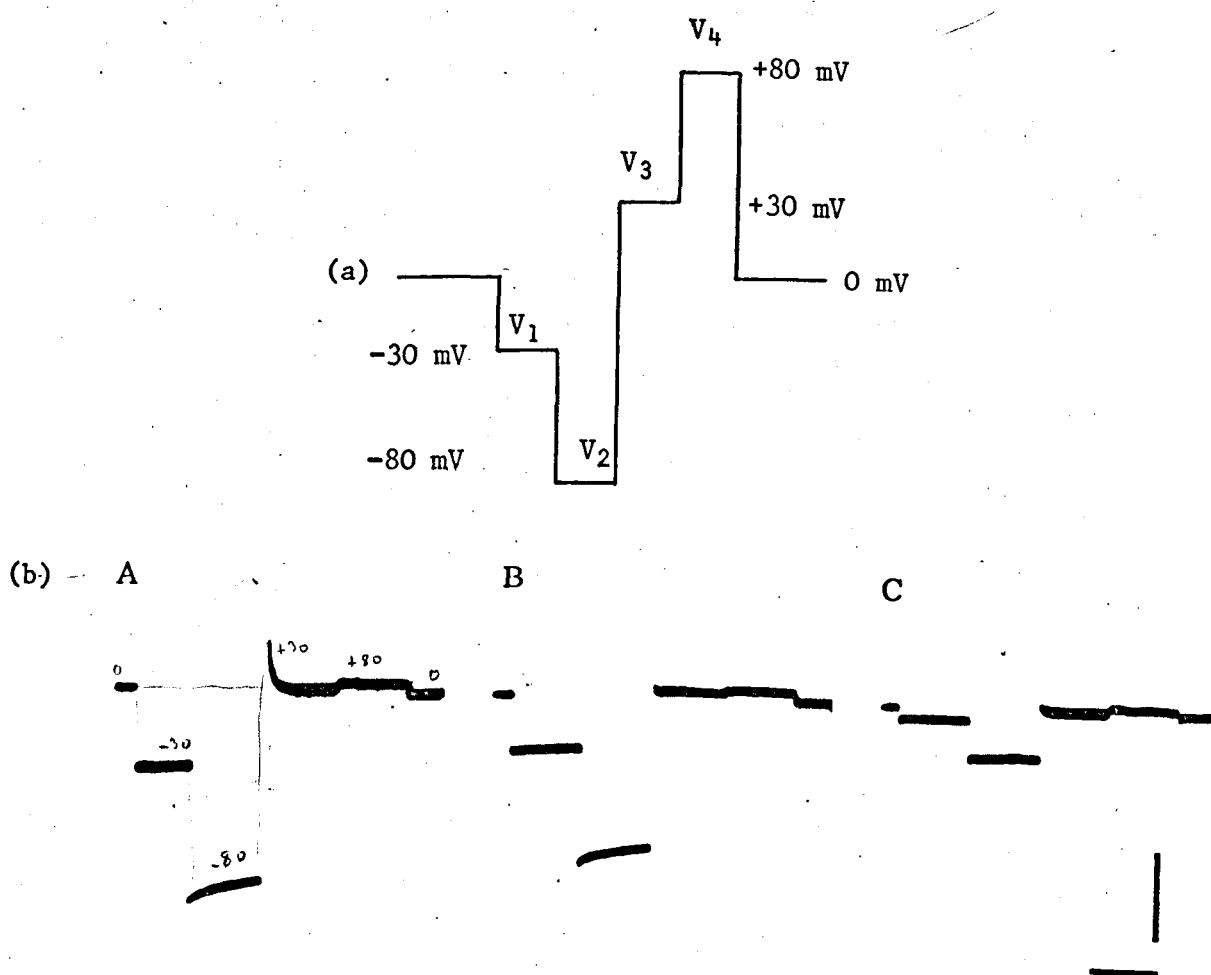


Fig. 49. The effect of 4-aminopyridine on the inward rectifier.

(a) Potential sequence $V_1 = -30$ mV; $V_2 = -80$ mV; $V_3 = +30$ mV; $V_4 = +80$ mV. (b) Section A: membrane currents in the absence of 4-AP. Section B: 30 min after addition of 5 mmole/l 4-AP pH 7.2 to bathing solution. Section C: 8 min after increase in extracellular pH to 9.8 still in 5 mmole/l 4-AP. Vertical calibration 0.2 mA/cm², horizontal calibration 500 msec. Fibre 15575, $l=100\mu$; $l'=50\mu$; $a=70\mu$; Temp. 19°C.

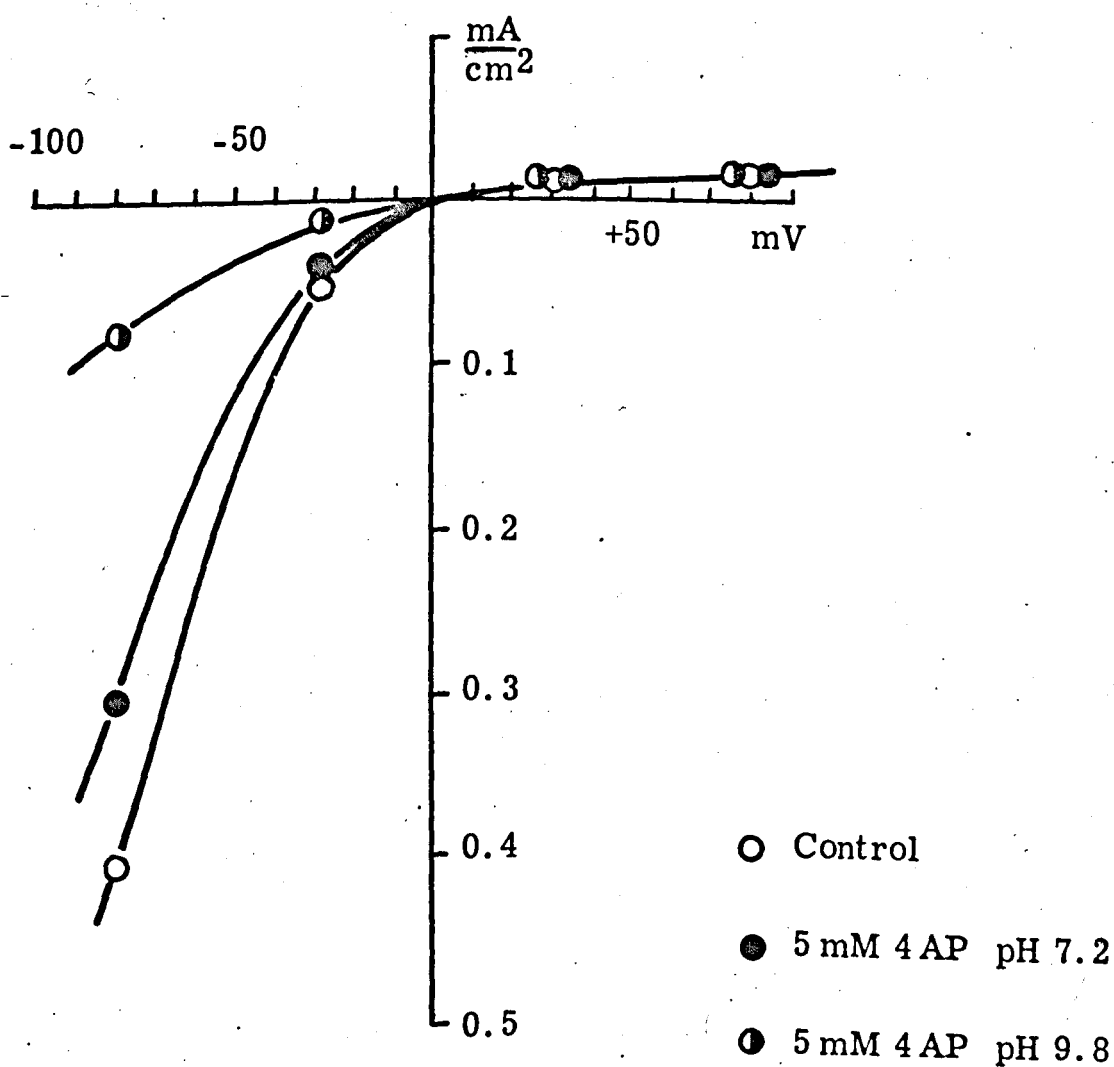


Fig. 50. Current Voltage plot of the data shown in Fig. 49

also block the inward current.

III. vi. Determination of the Membrane Capacity with the aid of 4-AP

Determination of the Membrane Capacity with the Aid of 4-AP

Fig. 51 shows the membrane current which flows upon depolarization in an artificially polarized fibre bathed in isotonic KCH_3SO_4 solution at pH 9.8. In the absence of 4-AP inward current flows through the delayed rectifier during a depolarization to -30 mV and outward current during a depolarization to $+30$ mV. After a 10 min soak in a solution containing 5 mmole/l 4-AP (pH 9.8) the delayed outward and inward potassium currents are abolished. The transient current which remains is associated with the charging of the membrane capacity, consequently does not change direction at the potassium equilibrium potential and the maintained residual outward current is the leakage current.

Such records can be used to estimate the membrane capacity, by determining the area under the transient portion of the curve. When this is done the membrane capacity was found to be $6.1 \pm 1.1 \mu\text{F}/\text{cm}^2$ (\pm S.D., $n = 6$). This value is similar to values already published in the literature (Katz 1948; Chandler, Rakowski and Schneider, 1976a).

Schneider & Chandler (1973) have detected a component of the membrane capacity in polarized fibres which they interpreted as an intra-membrane charge movement associated with the process of excitation contraction coupling. It was of interest to use such 4-AP treated muscles to explore the voltage dependence of the membrane capacity in this preparation and to investigate the action of formaldehyde on this charge movement, since formaldehyde interrupts the process of excitation contraction coupling.

Since this section is not directly related to the main theme of this thesis, consequently it will be given its own introduction, methods, results and discussion.

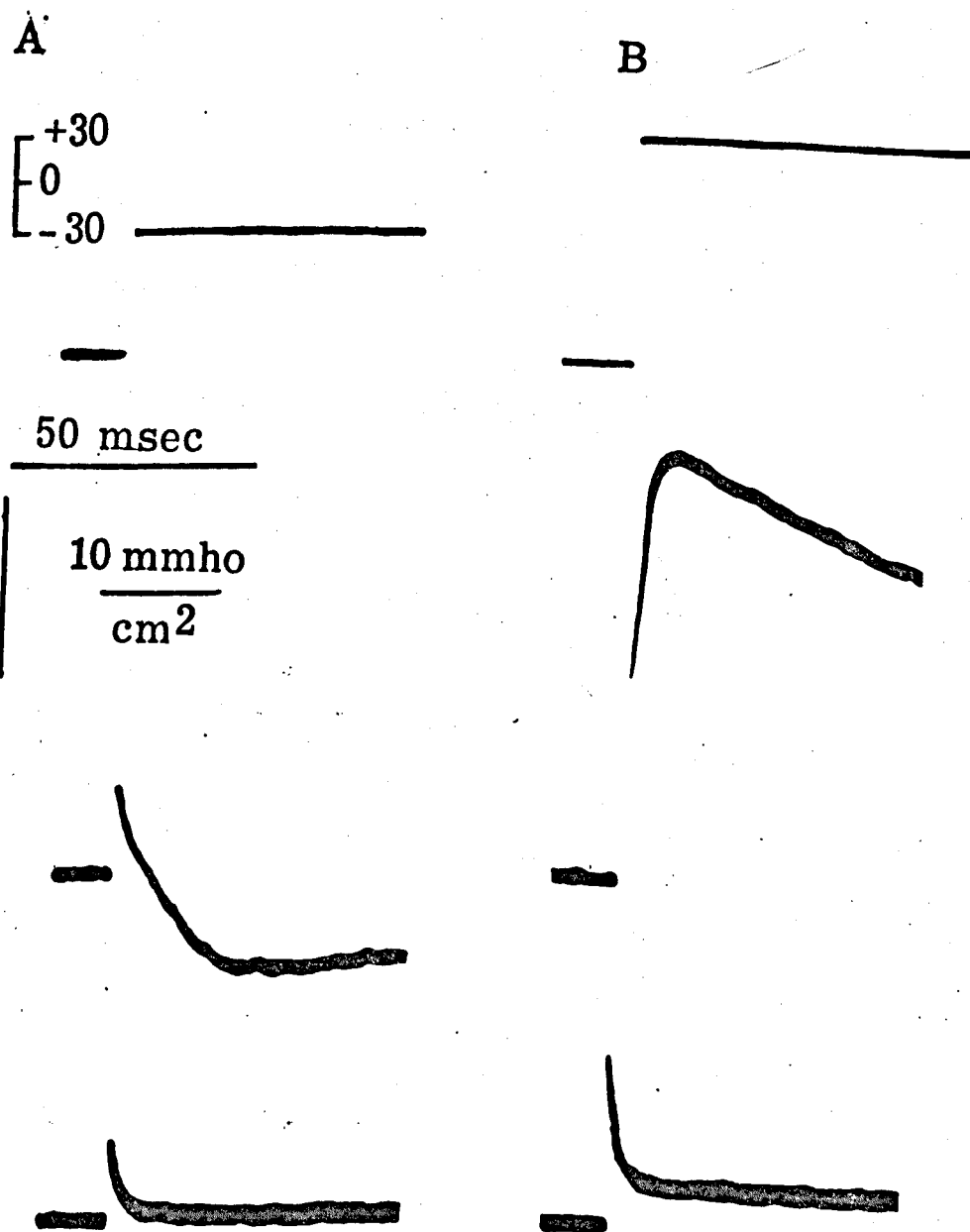


Fig. 51. Abolition of delayed inward potassium current (A); and delayed outward current (B) by 5 mmole/l 4-AP (pH 9.8). Top records, membrane potential; middle records, untreated delayed current; lower records, membrane current after 4-AP. Temp. 21°C. Fibre F19975; $l=200\mu$; $l'=50\mu$; $a=80\mu$.

III_{vii} • Asymmetries in the Membrane Capacity of Skeletal Muscle

Asymmetries in the Membrane Capacity of Skeletal Muscle

INTRODUCTION

Intramembrane Charge Displacement Currents in Skeletal Muscle

In 1973 Schneider & Chandler reported that the value of membrane capacity of a frog sartorius muscle fibre depended upon the voltage range in which it was determined. When determined from a voltage step from -79 mV to -29 mV the capacity was larger than when determined from a step from -144 mV to -79 mV. With the aid of a computer, to compare directly the capacity transients and to subtract one from the other, they were able to isolate the component which was responsible for the asymmetry in the capacity and to determine its time course. When this was done the component which remained was a small transient outward current which flowed upon depolarization and a transient inward current which flowed upon repolarization. Schneider & Chandler (1973) suggested that this current was the result of the re-orientation of charges or dipoles within the membrane as a consequence of the potential change. In keeping with this hypothesis they found that the amount of charge which moved at the beginning of the depolarizing step was exactly equal to that which returned during the repolarizing step, i.e. the 'on' charge movement equalled the 'off' charge movement.

In squid axons Armstrong & Bezanilla (1973) also recorded asymmetries in the membrane capacity. In nerve these charge movements were 1/3rd the magnitude of those in muscle and between 20 and 100 times faster. Armstrong & Bezanilla (1973) suggested that these currents in nerve were associated with the opening of the sodium channels. In muscle, however, the charge movement was too large and too slow to be associated with sodium channel gating; consequently, Schneider and Chandler proposed that they were associated with the process of excitation contraction coupling. They speculated that the charges of dipoles responsible were located at the

junction of the T-system and the sarcoplasmic reticulum and possibly associated with the feet like processes that occur at this location as seen in electron micrographs, (Franzini-Armstrong, 1970).

In 1976 Adrian & Almers (a & b), Chandler, Rakowski & Schneider (a & b) and Adrian, Chandler & Rakowski published papers which examined in much greater detail the properties of the charge displacement currents in polarized skeletal muscle bathed in hypertonic solutions.

After prolonged depolarization Chandler et al (1976b) found that the charge displacement was absent when looked for after a brief repolarization. This disappearance coincided with the muscle becoming mechanically refractory as had been described by Adrian, Chandler & Hodgkin (1969). Upon prolonged repolarization of the membrane both the mechanical response and the charge displacement current returned as determined with a subsequent depolarizing step and with a similar time course. Approximately 10% of the total charge displacement could be detected at the mechanical threshold. The voltage dependence of the amount of charge movement and the degree of mechanical activation were also found to have a similar form (Adrian, Chandler & Rakowski, 1976). In detubulated muscle fibres the link between excitation and contraction is interrupted (Fujino, Yamaguchi & Suzuki, 1961; Gage & Eisenberg, 1969). Chandler et al (1976b) found that detubulation also removed the majority of the charge displacement current. Taken together these findings support the earlier view of Schneider & Chandler (1973) that the charge displacement is associated with the process of excitation and contraction coupling.

In muscle fibres treated with 10 mM formaldehyde the mechanical response to electrical stimulation or to high potassium solution is abolished (Hutter, 1969, 1970; Hutter & Washio, personal communication). However, fibres can still be made to contract upon application of caffeine (Hutter & Washio, personal communication). This suggests that the abolition of the mechanical response is due to a localized action of

formaldehyde somewhere in the link between excitation of the membrane the release of calcium ions from the sarcoplasmic reticulum. It was therefore of some interest to investigate muscles bathed in isotonic KCH_3SO_4 for the presence of such a charge movement and to determine whether formaldehyde would have any effect on the charge displacement current.

METHODS

The experiments described here are much simpler than those described by Schneider & Chandler (1973) since no reliable means was available for averaging the signals. The membrane capacity transients obtained here were analysed manually at the end of each experiment by a method similar to that initially used by Keynes & Rojas (1974) on squid axons.

The membrane capacity was determined by measuring the area under the transient portion of the current record, either by projecting the current trace from the 35 mm film record on to mm² graph paper and counting the number of mm squares under the transient, or by assuming that the decay of the transient was an exponential process, then by determining the instantaneous value and the time constant of the decay. The effective capacity was then obtained from the expression:

$$C_{\text{eff}} = \frac{T_c}{2l^2 r_i \pi d} \left(\ln \frac{V_c}{V_o} \right) \quad 111.41$$

where l is the electrode spacing; r_i is the internal resistivity of the fibre; and V_o and V_c is the value of the instantaneous and the value at time $1/e$ for the decay process; T_c is the time constant of the decay;
; and d is the fibre diameter
 (See Adrian & Almers, 1976a).

The membrane capacity was determined over two different voltage ranges; firstly during a depolarizing step from a holding potential of -100 mV, and secondly, during a hyperpolarizing step of equal magnitude from the same holding potential. The difference in the membrane capacity during the 'on' and 'off' of each potential step was then determined.

All the experiments were done on 4-AP treated muscle fibres bathed in isotonic KCH₃SO₄ solution. Fibres were repolarized for at least 1 min before each measurement in order to reprime the charge (Chandler & ^{et al} 1976a). The bathing solution was made hypertonic with sucrose (500 mM) to reduce the mechanical response upon depolarization; all experiments were done at a

temperature in the range 6 - 10°C.

Formaldehyde solutions also contained 500 mM sucrose and were prepared immediately prior to use by the method already described. The concentration of formaldehyde used in all experiments was 15 mM.

RESULTS

Fig. 52 (a) shows diagrammatically the voltage protocol used in these experiments; (b) shows records of the membrane current before formaldehyde treatment and (c) after 30 min. exposure to formaldehyde. In section (b) the time course of the capacity transient recorded during the 'on' of the depolarizing step is different from that recorded during the hyperpolarizing step of the same magnitude. The area under the depolarizing 'on' transient is larger than the hyperpolarizing transient; similarly the area under the depolarizing 'off' transient is larger than the 'off' hyperpolarizing transient. After formaldehyde treatment the time course of the capacity transient is the same during the hyperpolarizing and depolarizing steps. It is likely that the asymmetry in the membrane capacity seen in (b) is similar to that reported by Schneider & Chandler (1973). The absence of any asymmetry after formaldehyde treatment suggests that the charge displacement currents are abolished by formaldehyde.

The difference in the membrane capacity determined at different membrane potentials for 8 fibres before and 6 fibres after formaldehyde treatment is shown in Fig. 53. In the untreated fibre the additional component of the membrane capacity becomes apparent at approximately -80 mV, its magnitude increases steeply with potential in the range -60 to -10 mV and approaches a limit as the potential is made more positive. In order to compare different fibres the additional component of the capacity, determined at intermediate voltages have been normalized to this maximum value. After treatment with formaldehyde there is little or no asymmetric component to the membrane capacity in the range -80 - +30 mV.

It is important to establish that the difference in the membrane capacity as seen in these KCH_3SO_4 , 4-AP treated fibres behaves in a similar fashion to that described by Schneider & Chandler (1973) on polarized fibres. A characteristic feature of the displacement currents in polarized fibres is that the amount of charge that moves during the 'on' is equal to that which

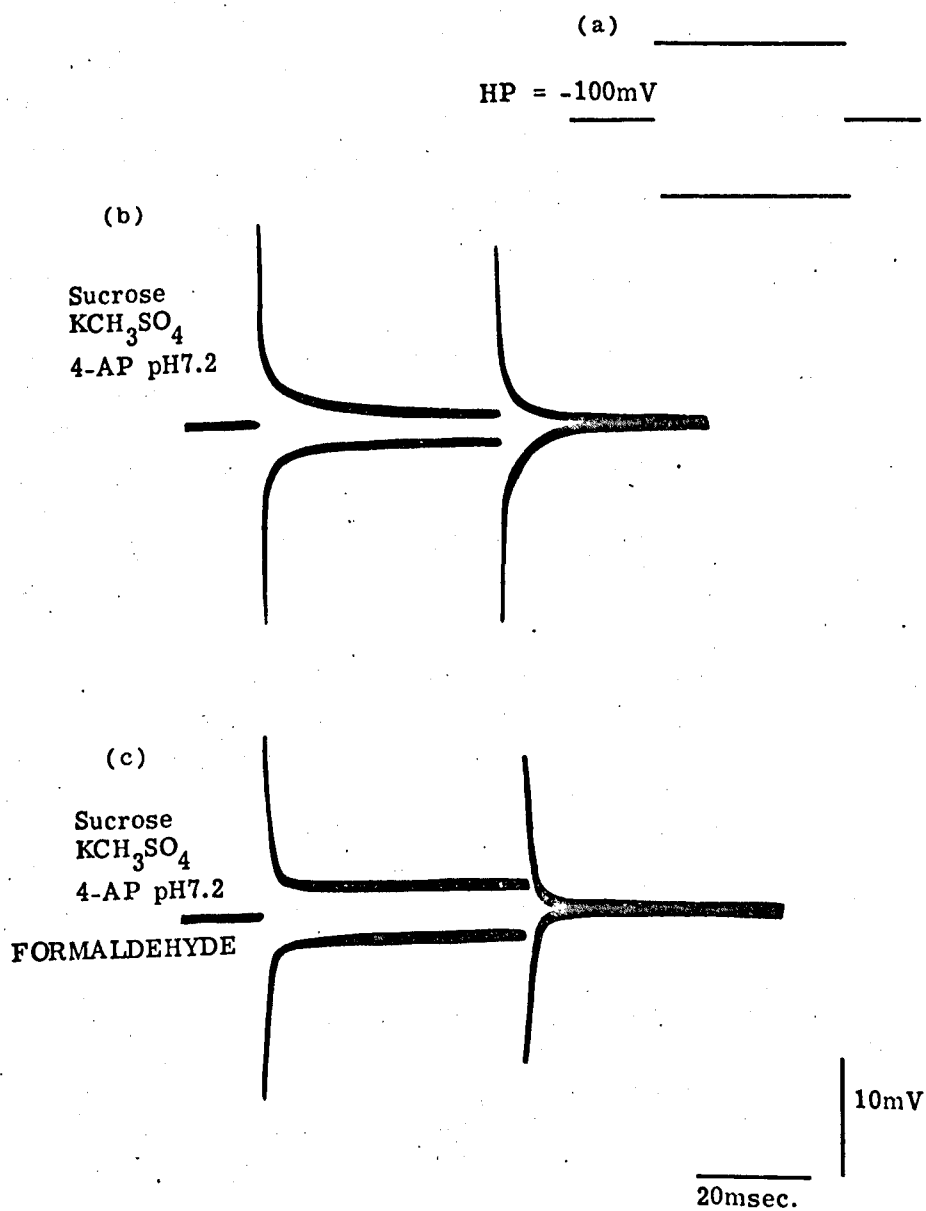


Fig. 52. The effect of formaldehyde on the membrane capacity.

(a) Schematic diagram of the voltage protocol; (b) Capacity transients in the absence of formaldehyde; Fibre F131875; $l=250\mu$; $l'=50\mu$; $a=80\mu$; Temp. 8°C . (c) Capacity transients after 30 min treatment with formaldehyde (15 mmole/l). Fibre F201975; $l=200\mu$; $l'=50\mu$; $a=70\mu$; Temp. 8°C .

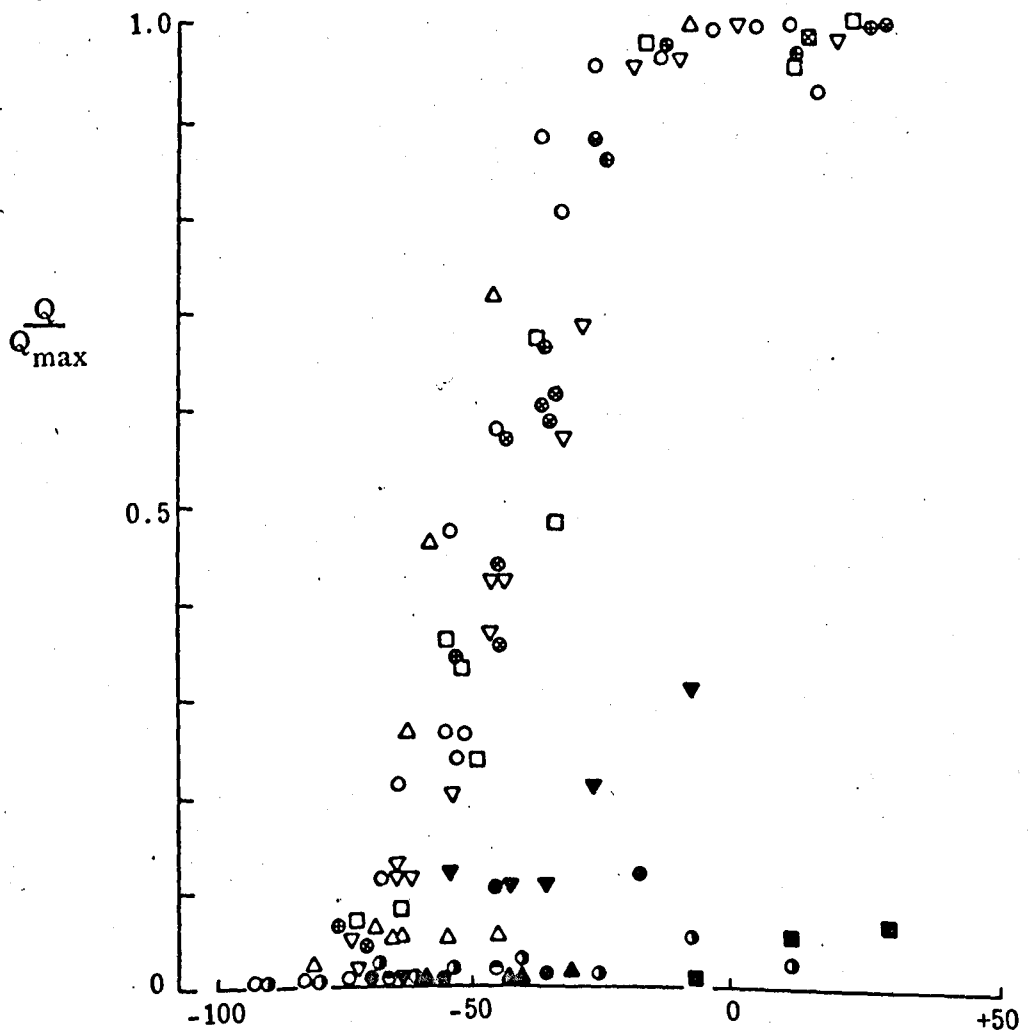


Fig. 53. The voltage dependence of the ratio Q/Q_{\max} for fibres before (open symbols) and after (filled or half filled symbols) formaldehyde treatment.

moves during the 'off' (Schneider & Chandler, 1973; Chandler et al, 1976; Adrian et al, 1976 a & b). Fig. 54 shows the results from three fibres in isotonic K^+ solution and plots the magnitude of the additional component of the membrane capacity determined during the 'on' against that determined during the 'off'. If the magnitude of the 'on' and 'off' components are equal the points should fall on a line of slope 0.5. For the fibres shown the 'on' component is approximately equal to the 'off'.

Another characteristic feature of the charge displacement current is its absence in fibres after prolonged depolarization and its reappearance after prolonged repolarization. Fig. 55 shows the magnitude of the asymmetry in the membrane capacity in a permanently depolarized fibre after repolarization steps of different durations. After a few msec repolarization there is no additional component to the membrane capacity in the depolarizing direction. With longer repolarizing steps the additional component returns and in this case reaches a maximum after approximately 60 sec repriming. This time course is similar to that reported by Chandler et al (1976b).

In Fig. 53 the additional component in the membrane capacity in untreated fibres can be fitted by an equation of the form:

$$\frac{Q}{Q_{\max}} = (1 + \exp^{-\frac{(V - \bar{V})}{k}})^{-1} \quad 111.42$$

where Q is the amount of charge moved at a voltage V ; Q_{\max} is the maximum amount of charge that can move; \bar{V} is the voltage where $Q/Q_{\max} = 0.5$; and k determines the voltage sensitivity of the relation. Table 6 presents the results from 8 fibres analyzed in terms of equation III.42. Schneider & Chandler (1973) and; Chandler^{et al} (1976a) and Adrian et al (1976) have fitted their results to a similar equation. Their values for Q_{\max} , \bar{V} and k are also shown in Table 6. Values obtained using 4-AP and high K treated fibres agree reasonably well with the published data on the charge displacement currents.

Table 7 shows the results from 6 fibres after formaldehyde treatment. In each case the additional component of the membrane capacity is reduced.

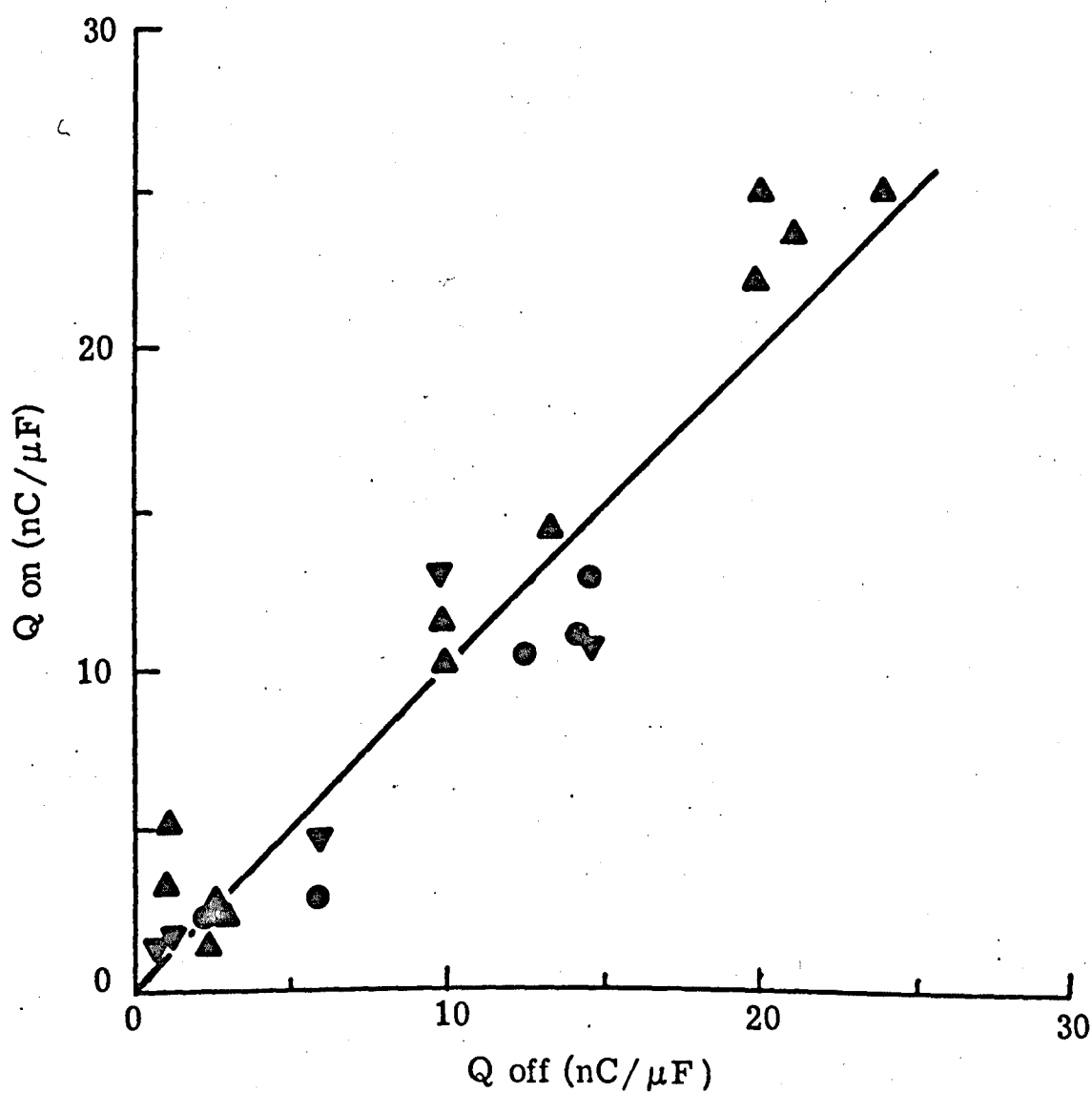


Fig. 54. The effect of membrane potential on the asymmetry in capacity: Comparison of the areas of the 'off' and 'on' components.

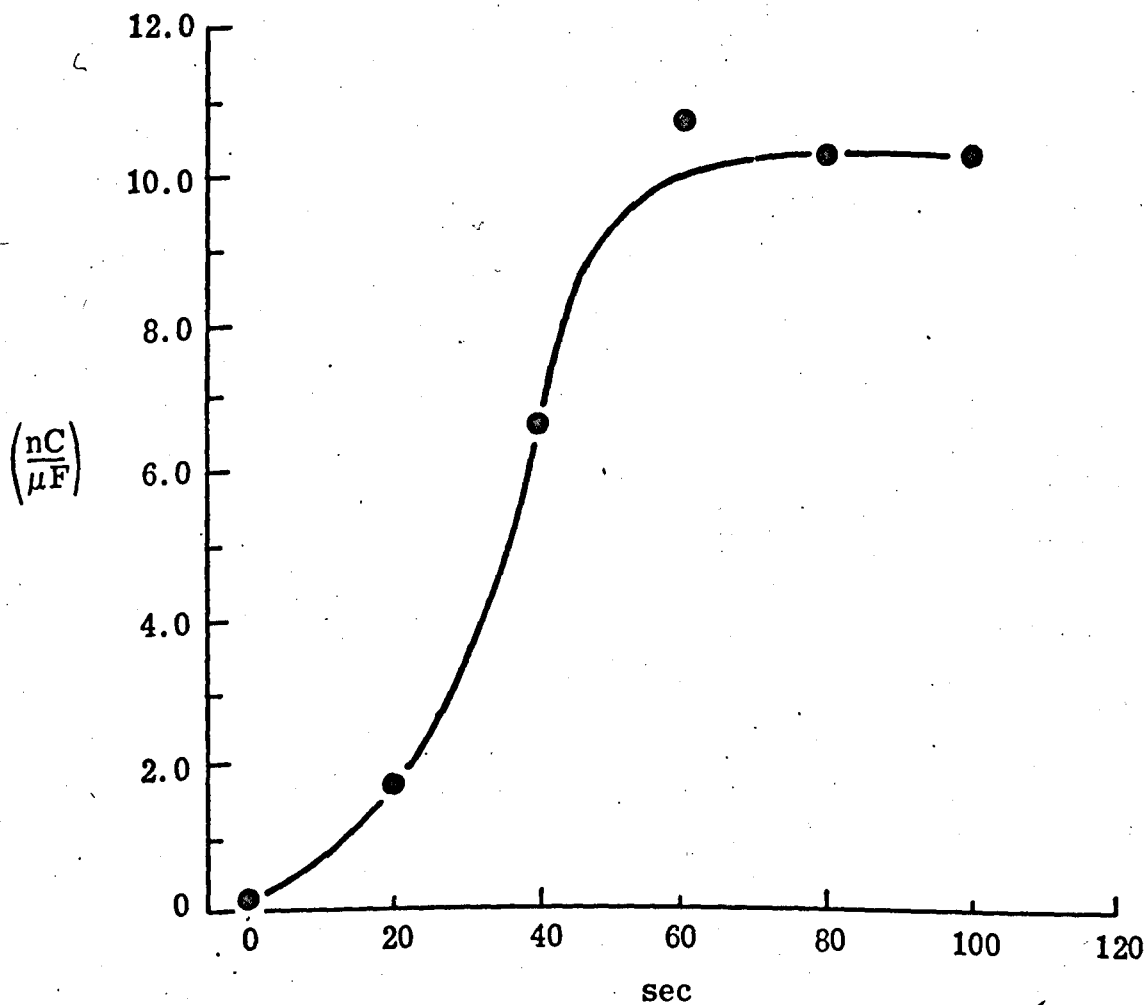


Fig. 55. Repriming curve for the asymmetry in membrane capacity. The time shown on the abscissa is the duration of the repriming step to -122 mV. Fibre F1 11576: $l=525\mu$; $l'=50\mu$; $a=80\mu$; Temp. 8°C ; pH 7.3. This experiment was done in the presence of 10 mmole/l RbCl to abolish any residual inward rectification. Curve drawn by eye.

TABLE 6

HYPERTONIC KCH₃SO₄

	<u>Q_{max}</u>	<u>\bar{V} (mV)</u>	<u>k (mV)</u>
F1 31875	31.0 nC/ μ F	-53	8.0
F1 29875	32.5 mVmsec	-57	8.2
F1 11576	32 nC/ μ F	-49	12.4
F2 01975	17.5 mVmsec	-48	12.9
F3 11975	20.0 mVmsec	-42	14.9
F1 01875	17.5 mVmsec	-46	14.9
F3 12975	16.0 mVmsec	-40	11.2
F1 11975	20.0 mVmsec	-40	14.8

HYPERTONIC RINGER

	<u>Q_{max}</u>	<u>\bar{V} (mV)</u>	<u>k (mV)</u>
Schneider and Chandler (1973)	25 mVmsec	-44	8
Chandler, Rakowski and Schneider (1976)	25 nC/ μ F	-44	8
Almers and Best (1976)	24.5 nC/ μ F	-44.1	12.6
Adrian and Almers (1976)	32.0 nC/ μ F	-48.6	7.8

TABLE 7

FORMALDEHYDE TREATED

	<u>Q_{max}</u>	<u>\bar{V} (mV)</u>	<u>k (mV)</u>
F2 01975	2 mVmsec	-	-
F5 11975	3 nC/ μ F	-	-
F4 11576	1.5 mVmsec	-	-
F3 29875	10 nC/ μ F	-	-
F1 31875	1.5 mVmsec	-	-
F3 11576	0	-	-
Almers & Best (1976)	16.5 nC/ μ F	-31.5	16.8

DISCUSSION

It may be tentatively concluded that the asymmetry in the membrane capacity seen in these experiments is the same as that recorded by other workers. It would also appear that this asymmetry is abolished by formaldehyde treatment which tends to support the idea that the asymmetry is associated with the process of excitation contraction coupling. On the other hand if it is accepted that the asymmetry is associated with E.C. coupling then the results point to the site of action of formaldehyde in the abolition of the mechanical response which follows electrical stimulation.

Chandler & Schneider (1976) have pointed out that asymmetries in membrane capacity would arise as a result of inward rectification. In the presence of an inwardly rectifying component of the membrane current the membrane time constant, R.C, would be larger during depolarizing steps than during hyperpolarizing steps. Since weak solutions of formaldehyde also abolish inward rectification it is to be expected that an asymmetry in capacity on this basis would be abolished. The possibility must therefore be considered that in the present experiments the asymmetry in capacity is the result of residual inward rectification and that the abolition in the asymmetry is the effect of formaldehyde on this residual component.

Two points argue against such a possibility. First, the inward rectifier is almost completely abolished in fibres treated with 5 mM 4-AP in alkaline solution. Second, in experiments done also in the presence of 10 mM rubidium to further reduce any residual inward rectification the asymmetry in the membrane capacity could still be detected. It therefore seems likely that inward rectification plays no part in these experiments.

Almers & Best (1976) have recently reported that they could find little or no effect of 10 mM formaldehyde on directly recorded charge displacement currents, although they previously reported a 50% reduction in Q_{\max} (Almers, Adrian & Levinson, 1975). Almers & Best (1976), however,

did find that the voltage sensitivity of the relation describing the amount of charge moved at different voltages is less steep than in untreated fibres. In these experiments, as shown in Fig. 53, the difference in the magnitude of the membrane capacity after formaldehyde is reduced to a much greater extent.

The reduction in Q is such that it is impossible to fit equation III.42 to the present results, but it appears likely that the parameter k is increased to a greater extent in these experiments than those of Almers et al (1976, 1977). The reason for the difference in the potency of formaldehyde in reducing the charge displacement current is unknown at present.

III viii. The Effects of pH on the Delayed Potassium Currents

The Effects of pH on the Delayed Potassium Current

In Section III₁, the actions of 4-AP were examined on the delayed potassium currents at different extracellular pH. Before these experiments were done the effect of pH alone was examined on the delayed currents. The results from these pH experiments are described below.

As can be seen in Fig. 56, in acid and alkaline solution the delayed potassium current continues to function. The currents activate along a sigmoid time course to reach a peak and then decay with an approximately exponential time course to zero. Fig. 57 shows a semilogarithmic plot of the voltage dependence of the peak conductance at pH 7.2, 5.0 and 9.8. At each pH the data has been normalized to the maximum available conductance; and fitted by the equation:

$$\frac{g_K}{\bar{g}_K} = \left(1 + \exp - \frac{(V - \bar{V})}{k}\right)^{-1} \quad 111.43$$

where V is the applied electric field; \bar{V} is the voltage of half maximal activation and k describes the voltage sensitivity of the relation in its most sensitive range. The parameters \bar{V} , and k for this fibre (10.3.75II) are shown in Table 8; the results from 7 other fibres are also shown. At both pH 5.0 and 9.8, \bar{V} is shifted to the right by 19.5 mV. The value of k is not markedly affected by pH but in all fibres the lowest value of k is at neutral pH.

The maximum potassium conductance \bar{g}_K , is reduced in both acid and alkaline solution. The extent of this reduction varies considerably from fibre to fibre as can be seen from column III, Table 8.

In two experiments the voltage dependence of the steady state inactivation (k_∞) was determined at pH 7.2, 9.8 and 5.0 in the same fibre. Fig. 58 shows the results from one of these experiments. The protocol for these experiments was the same as that described in section III₁ for the effect of 4-AP on k_∞ . At each pH the inactivation curve could be fitted by

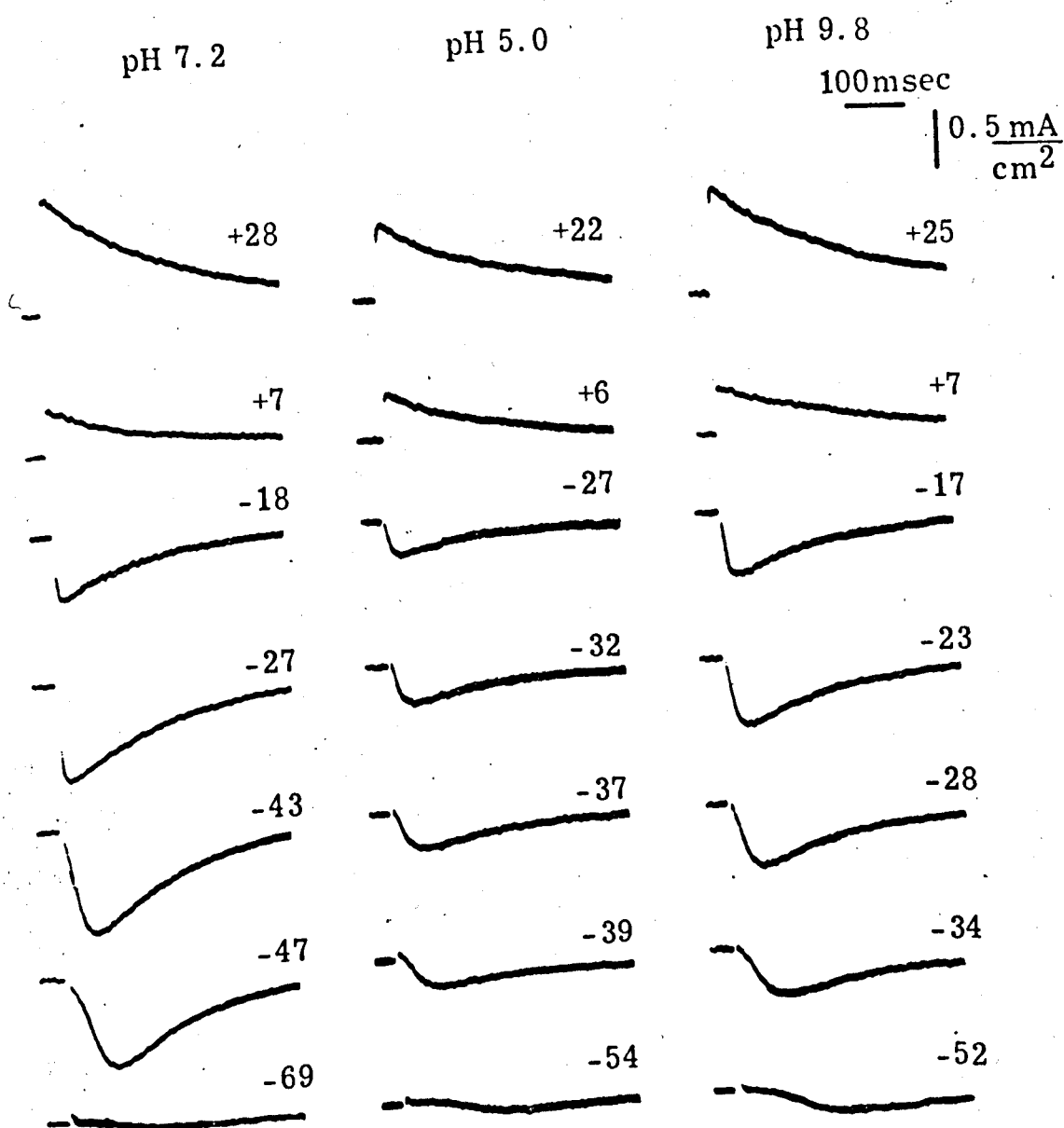


Fig. 56. The effect of pH on the delayed potassium currents in a formaldehyde treated fibre bathed in isotonic KCH_3SO_4 solution. Fibre 14.0376, $l=150\mu$; $l'=50\mu$; $a=80\mu$. Temp. 21°C .

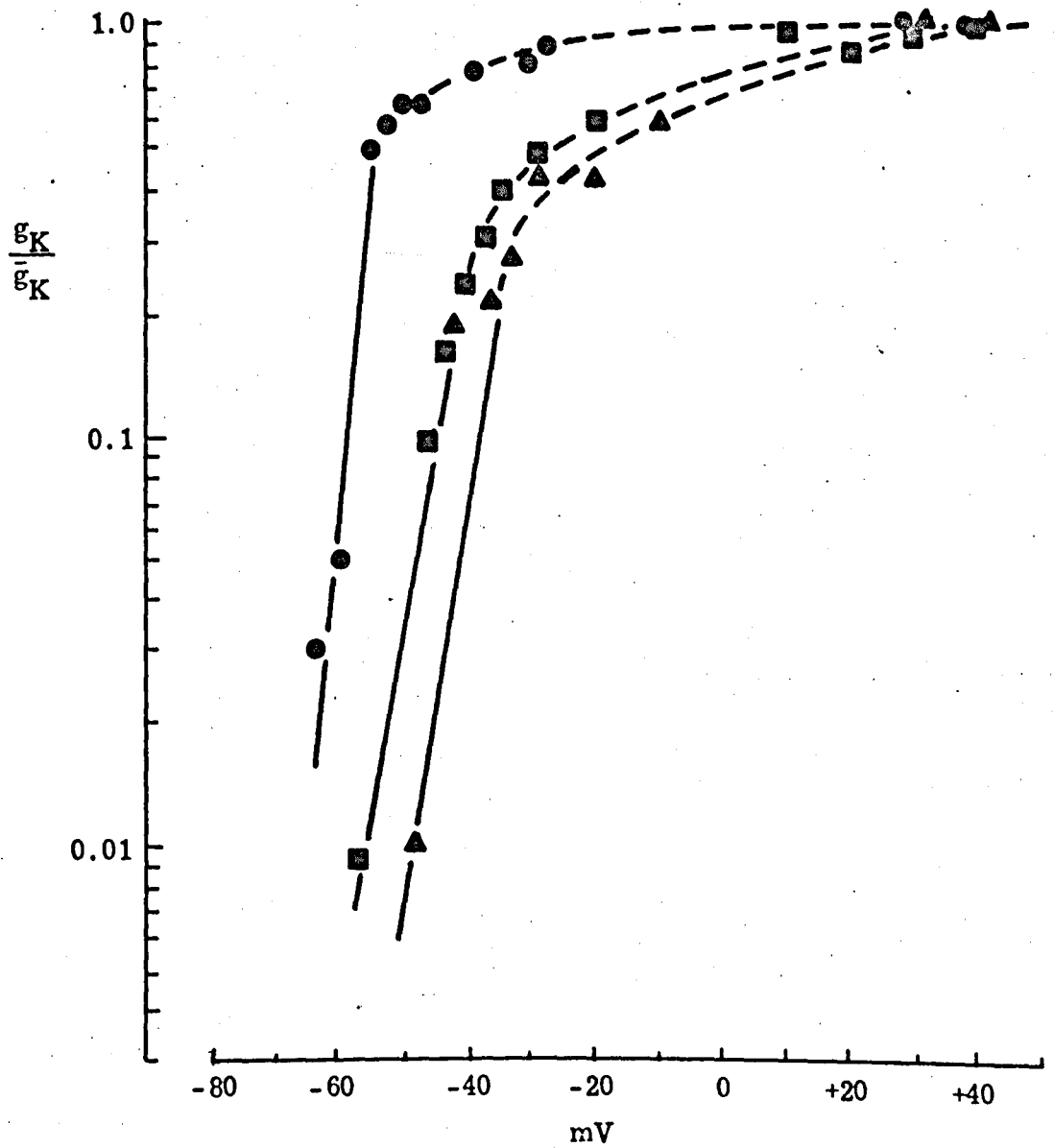


Fig. 57. The voltage dependence of the peak potassium conductance at different extracellular pH.

Fibre 10.3.75 II: pH 5.0 (▲); pH 7.2 (●); pH 9.8 (■);
 $l=250\mu$; $l'=50\mu$; $a=80\mu$; Temp. 19°C .

TABLE 8

The effect of pH on the delayed rectifier

FIBRE	pH _o	\bar{g}_K	\bar{V}	$\Delta\bar{V}$	k
10.3.75 II	7.2	20.7	52.5	-	5.1
	5.0	20.4	26.0	+26.5	6.3
	9.8	20.2	20.0	+32.5	7.0
14.3.75 I	7.2	34.5	40.0	-	6.6
	5.0	30.0	20.0	+20.0	8.3
	9.8	28.3	32.0	+ 8.0	8.5
14.3.75 II	7.2	40.8	40.1	-	7.6
	5.0	32.1	18.0	+22.1	8.3
	9.8	31.0	33.5	+ 6.5	8.6
2.10.75 I	7.2	12.4	58.0	-	7.0
	5.0	10.9	37.0	+21.0	7.0
	9.8	11.2	37.0	+21.0	7.0
10.3.75 I	7.2	46.3	32.0	-	8.0
	5.0	34.7	8.0	+22.0	8.0
18.3.75 I	7.2	33.8	44.5	-	7.6
	5.0	28.8	35.0	+ 9.5	7.6
18.3.75 II	7.2	34.0	53.0	-	8.0
	9.8	28.0	43.0	+10.0	8.0
7.10.75 I	7.2	29.1	53.0	-	4.0
	9.8	27.0	41.0	+12.0	6.3

TABLE 9

	pH	$\bar{V}_{K\infty}$	\bar{V}_n	$\Delta V_{K\infty}$	$\Delta V_{n\infty}$	δ
14.4.75 I	7.2	46	40	-	-	8.0
	5.0	28	19	18	11	8.0
	9.8	40	32	6	8	8.0

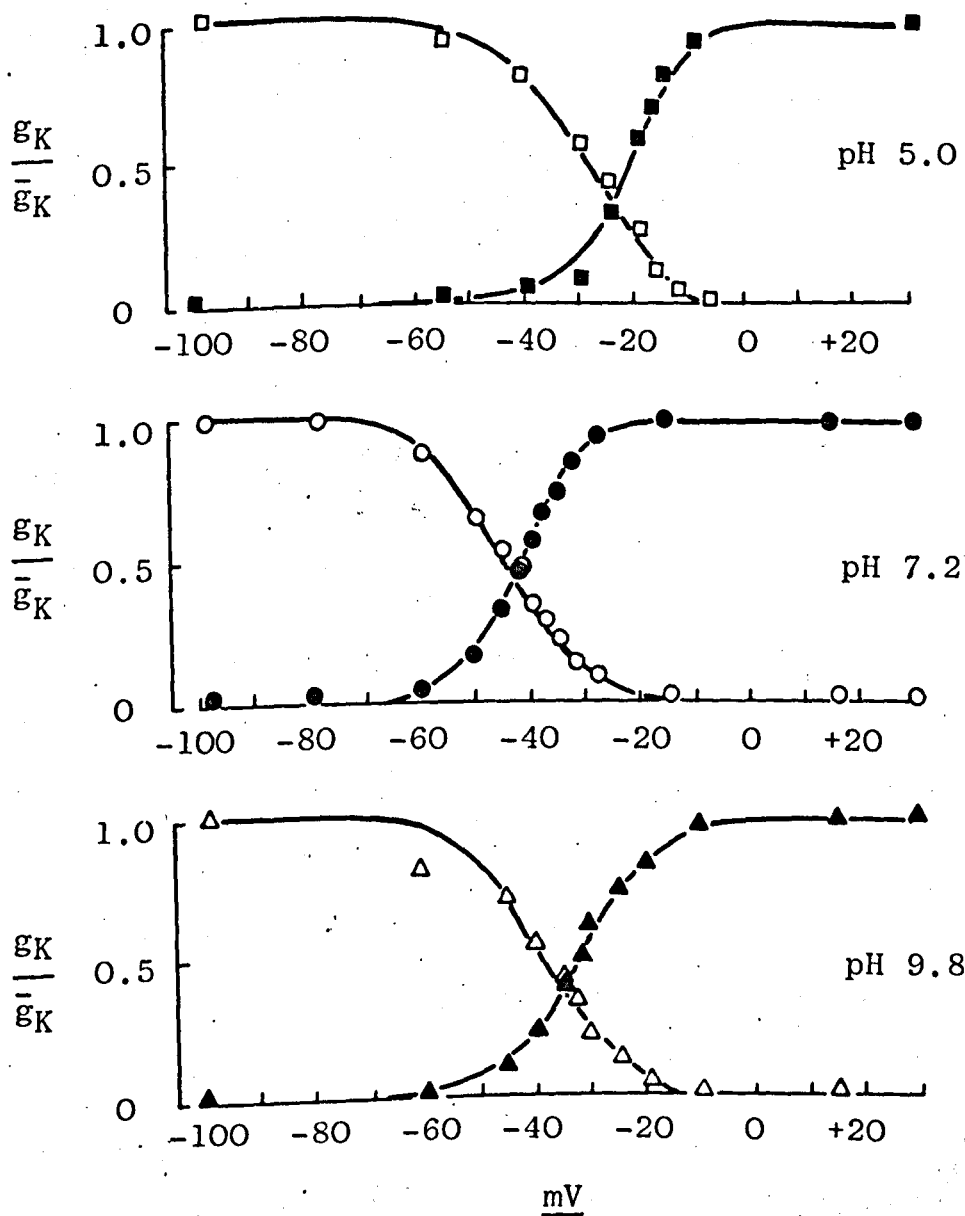


Fig. 58. The voltage dependence of the peak potassium conductance (filled symbols) and the steady state inactivation curve (open symbols) at different extracellular pH: Fibre 144751; $l=150\mu$; $a=70\mu$; Temp. 19°C .

an equation of the form

$$k_{\infty} = (1 + \exp - \frac{(V - \bar{V})}{\delta})^{-1} \quad 111.44$$

and the parameters $\bar{V}_{k_{\infty}}$ and \bar{V}_n are shown in Table 9. Both $\bar{V}_{k_{\infty}}$ and \bar{V}_n are shifted to more positive potentials and by similar amounts; and δ is not affected. Since, both n and k curves are shifted in the same direction and by similar amounts a possible interpretation of this situation would be that pH modifies the membrane surface potential thereby altering the total transmembrane potential.

The time course of inactivation was examined at the different pH and at different membrane potentials. In every case the decay could be fitted by a single exponential. Fig. 59 shows a plot of the voltage dependence of the rate of inactivation at each pH. As in neutral solution, the rate of inactivation does not vary systematically with potential at pH 5.0 and 9.8; and has values in the range 0.004 msec^{-1} to 0.009 msec^{-1} . Thus the extremes of pH used in these experiments have little effect on the delayed rectifier.

Hille (1969), Drouin and The (1969) and Woodhull (1972) have examined the effects of pH on the kinetics and maximum conductance of sodium and potassium channels in nerve. In general they all found that an increase in the hydrogen ion concentration shifts the voltage dependence of the sodium and potassium activation curves and the sodium inactivation curve in the depolarizing direction. An increase in the hydrogen ion concentration also reduces the maximum sodium and potassium conductance. These results in nerve are similar to those found here in skeletal muscle.

In nerve a decrease in the extracellular hydrogen ion concentration causes a shift in the activation and inactivation curves in the hyperpolarizing direction. This is the opposite to what happens in skeletal muscle where it is found that the n and k curves are shifted in the

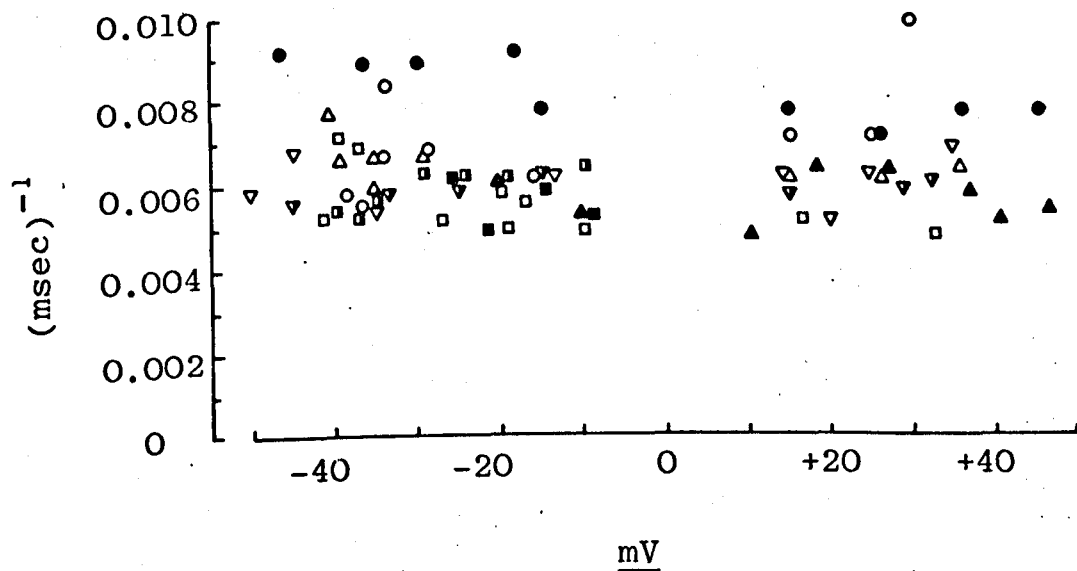


Fig. 59. The rate constant of inactivation of the delayed potassium current in solutions of different pH; pH 5.0, filled symbols; pH 7.2, open symbols; pH 9.8 half filled symbols. Temp. 20 - 24°C.

depolarizing direction. At present there is no adequate explanation for this result.

The shifts that are observed probably reflect changes in the membrane surface potential whilst the reduction in the maximum conductance is caused by a blockage of the channels (Hille 1969; Woodhull 1972). This blockage probably occurs at acidic sites within the channels which become associated with the hydrogen ion.

In the course of these experiments it was noted that the reduction in maximum potassium conductance that usually occurred in acid solution was apparently dependent upon potential or on activity in the fibre. In fibres that were left in the depolarized state during exposure to acid solution it was found that the magnitude of \bar{g}_K when determined after suitable repriming fell during successive measurements. Fig. 60 shows how \bar{g}_K changed at pH 7.2, 6.6 and 5.0 with a series of depolarizing steps; the interval of time between steps is 20 sec. In neutral solution there is little change; at pH 6.6 after a transient increase, which was seen in most fibres, \bar{g}_K was diminished subsequently to approximately 80% of its initial value after 14 test steps. At pH 5.0 the reduction in \bar{g}_K developed much quicker and to a greater extent. As to whether this blockage of the delayed potassium current was dependent on potential and time or on activity was not investigated. A similar use dependent block of the potassium channel was observed in depolarized fibres bathed in TEA solution.

In seeking an explanation for these results in acid and in TEA solution it may be suggested that the use dependent block is a consequence of the channels being in the inactivated state during the application of the blocking agent. In the inactivated state it is possible that the H^+ and TEA^+ ions cannot reach their blocking sites. Upon repolarization, inactivation is removed thereby uncovering the blocking site and the block is able to proceed. This interpretation is in keeping with the model of the outer section of the channel as suggested by the blocking actions of $4-AP^+$.

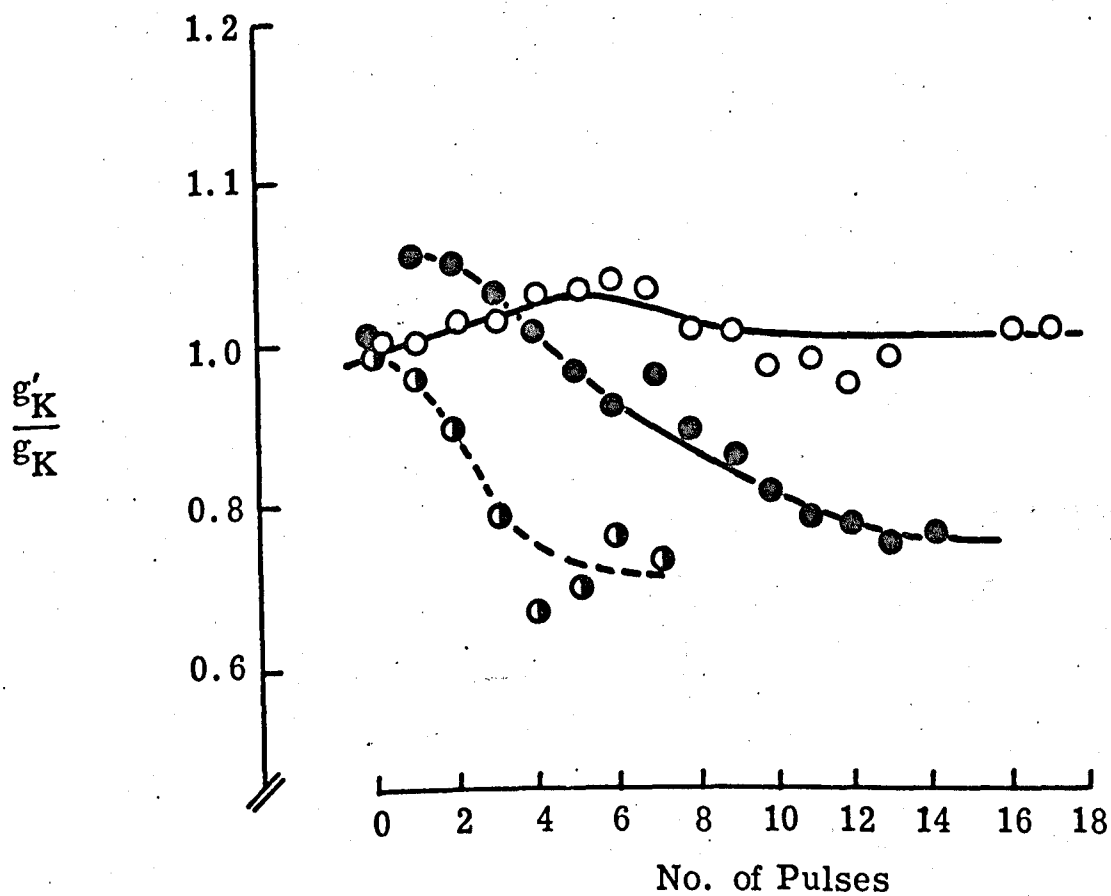


Fig. 60. The effect of repetative pulsing on the peak potassium conductance at different pH in fibres bathed in isotonic KCH_3SO_4 solution. Open symbols: pH 7.2; filled symbols: pH 6.6; half-filled symbols: pH 5.0.

These results with extracellular H^+ and TEA ions may also be taken as support for the idea that the inactivation mechanism of the potassium channel in skeletal muscle is located close to the outer surface of the membrane.

IV. DISCUSSION

IV._i Blockage of Potassium Channels by 4-aminopyridine

Comparison of the blocking action of 4-AP in nerve and muscle

Whilst the present work on skeletal muscle was in progress several reports have appeared in the literature regarding the mechanism of 4-AP blockage of the delayed potassium channel in squid and frog nerve (Meves & Pichon 1975, 1977 a & b; Yeh, Oxford, Wu & Narahashi 1976 a & b; Ulbrecht & Wagner 1976). These experiments confirm the findings of Pelhate & Pichon (1974) that 4-AP is a potent and selective blocking agent for the potassium channel in nerve and in addition, reveal that the blockage in nerve varies with voltage and time.

In the presence of 4-AP the switch on of the potassium current in squid axons and frog node of Ranvier is significantly slowed; and obeys simple first order kinetics. This slowing becomes less pronounced during repetitive depolarizing steps (Meves & Pichon, 1977a; Ulbrecht & Wagner, 1976; Yeh et al, 1976 a & b). By way of explanation it is proposed that 4-AP binds to a blocking site within closed channels. Upon depolarization both blocked and unblocked channels undergo identical transitions into the 'open' state but the blocked channels are not able to conduct potassium ions. However with time, under the influence of the membrane electric field 4-AP cations are slowly displaced from their blocking sites and the channel able to conduct potassium ions. The switch on of the potassium current in 4-AP treated nerve is thus determined by the kinetics of 4-AP release. Upon repolarization the channels close normally and 4-AP tends to return to its blocking position. The rate of return is much slower than the rate of release, consequently during a subsequent depolarizing step fewer channels will be initially in the blocked state and the turn on of the potassium current will be more normal.

In frog node of Ranvier, Ulbrecht and Wagner (1976) found that the blocking action of 4-AP on the steady state potassium current was less

pronounced at more positive membrane potentials; however, complete reversal of the block, even at low concentrations (13-212 μM), was never observed. This result has been interpreted by Ulbrecht & Wagner to mean that 4-AP has two blocking actions on frog node. One action is a voltage and time dependent decrease in the level of block upon strong depolarization, which they call the 'phasic' block and the other is a voltage and time independent block or 'tonic' block which accounts for the persistence of the block at positive potentials. The level of the 'tonic' block was found to vary with the concentration of 4-AP in a dose dependent manner. In squid axons Meves & Pichon (1977a) and Yeh et al (1976 a & b) also found that the blocking action of 4-AP on the steady state potassium current, as determined at the end of a 50 msec depolarizing step, was less pronounced at positive potentials. In squid, however, a strong depolarization to +80 mV was able to completely reverse the blockage caused by 0.1 mmol/l 4-AP (Meves & Pichon, 1977b).

Meves & Pichon (1977a) and Yeh et al (1976 a & b) have examined the block caused by 4-AP present in the intracellular and extracellular solutions on perfused squid axons. They found that it did not matter whether 4-AP was on the outside or inside of the membrane, the block was the same.

It would appear at first sight that the blocking actions of 4-AP in nerve are totally different from those of 4-AP in skeletal muscle. However if one compares only the blocking actions of 4-AP_o^+ in muscle with those in nerve there are some similarities: (a) an intensification of the block at more negative membrane potentials, and its partial relief upon strong depolarization; (b) the separation of the overall block into a voltage dependent and a voltage independent component.

Ulbrecht & Wagner (1976) describe the voltage dependence of the steady state blockage of the potassium channel in frog node with the

expression:

$$\frac{1}{r_{\infty}} = 1 + C_1 + C_2 \exp\left(-\frac{(\delta EF)}{RT}\right) \quad \text{IV.1}$$

where r_{∞} is the fraction of channels not blocked by 4-AP at a membrane potential E ; C_1 and C_2 are constants which depend on the concentration of 4-AP; and δ is the fraction of the applied membrane electric field 'seen' by the voltage dependent blocking site. A similar expression has been used in this thesis (equation III.36) to describe the steady state blocking actions of 4-AP^+ in skeletal muscle. Table 9 shows the parameters of equation III.36 compiled from the data on each tissue which describes the voltage dependence of the steady state blockage. In addition Table 9 also shows the rates of onset and offset of 4-AP blockage.

The dissociation constant for the voltage independent blocking action of 4-AP is shown in row 1 of Table 9. For some, as yet unexplained, reason 4-AP is approximately 10 times more effective on frog nerve than on skeletal muscle. The voltage independence of this blockage suggests that in both frog nerve and muscle this site lies outside the electric field, probably on the outer surface of the membrane. In squid axon there is no 'tonic' block; this suggests either that there is no appropriate binding site for small cations on the outer aspect of the channel or that 4-AP cannot gain access to this site. It is likely that the former explanation is correct since Armstrong & Binstock (1966) came to a similar conclusion on the basis of their finding of no blockage of the potassium current in the presence of TEA ions in the extracellular solution. In squid axon therefore C_1 in equation IV.1 can be assigned a value of zero.

The voltage dependent component of the block suggests that the binding site lies within the electric field; the fraction of the applied electric field seen by the site ' δ ' is shown in row 2. In each tissue δ has approximately the same value of 0.67; if the field within the membrane

TABLE 9

COMPARISON OF THE MODE OF ACTION OF THE AMINOPYRIDINES ON
NERVE AND SKELETAL MUSCLE

	<u>SQUID AXON</u>		<u>FROG NODE</u>	<u>MYXICOLA AXON</u>	<u>FROG MUSCLE</u>
	4-AP Narahashi et al (1976)	4-AP Pichon & Meves (1977)	4-AP Ulbrecht et al (1976)	2-AP Schauf et al (1976)	4-AP (Loc cit)
(K _{diss}) _t mM	-	-	0.16	0.36	1.1
δ	-	0.38 - 0.63	0.77	0.82	0.67
(T)release (~0mV)	~3sec (1mM)	6-30 msec (100 μ M)	200 msec (53 μ M)	<<msec (1 mM)	<<msec (1 mM)
(T)return (~ -80mV)	~27sec (1mM)	22sec (100 μ M)	60sec (53 μ M)	<<msec (1 mM)	5-10msec (1 mM)

is linear then the blocking site lies approximately $2/3$ the way across the channel from the outside.

A striking difference between the blocking action of the aminopyridines in nerve and 4-AP^+ in skeletal muscle is the rate at which 4-AP arrives and leaves the internal blocking site. The rates for particular concentrations of 4-AP and particular voltages are shown in row 3 of Table 9. In muscle the release of 4-AP was not directly observed, however the turn on of potassium current upon depolarization is unchanged in the presence of 4-AP^+ which suggests that the release may be complete before the channels open. The release in muscle must therefore be in the order of msec or fraction of a msec. In nerve the time constant for the release is much slower and is several hundred msec. The rate of onset of the block in nerve is also much slower than in muscle; the time constant being larger by 3 to 4 orders of magnitude.

A possible explanation for these similarities and differences may lie in the molecular architecture of the potassium channel in the different tissues. A possible clue to the structure of the channels may be the observation that in nerve 4-AP has the same blocking action when applied on either the outside or inside of the membrane. This result suggests that 4-AP can pass through the potassium channels in nerve. For example, 4-AP applied to the inside of the membrane will block the channel and strong depolarization will relieve the blockage presumably by pushing 4-AP through towards the outside. A difficulty with this idea is that the 4-AP molecule is apparently too large to pass the narrowest point in the channel. In nerve the diameter of the channel at its narrowest point is approximately 3\AA (Hille, 1967) whilst the 4-AP molecule has dimensions $4 \times 6\text{\AA}$ and is as thick as the benzene ring (Christen et al, 1975). In order to account for this anomalous situation it may be proposed that the lipophilic 'tail' of the 4-AP molecule becomes embedded in the channel wall; this would effectively reduce the diameter of the molecule

to that of the polar head. The HN^+ group of the 4-AP molecule is approximately 3 \AA and may just squeeze through the narrow section of the channel. This embedding of the lypophylic tail may also provide an explanation for the slow rates of removal and return of the block in nerve. 4-AP does not pass through the potassium channel in muscle possibly because the effective diameter of the molecule may not be reduced by the embedding of the lypophylic tail, consequently movement within the channel will not be hindered.

If this interpretation of the experimental results is correct then in nerve the composition of the channel wall at its outer aspect and in the narrow section is predominantly lypophylic in nature whilst in skeletal muscle the same sections of the channel are more hydrophylic.

Yeh et al (1977 a & b) have also examined the blocking actions of 2-AP on the potassium channel of squid axons and have found the mode of action to be essentially the same as that of 4-AP. Schauf et al (1976) have reported that the blocking actions of 2-AP on the potassium currents in *Myxicola* axons appears to be different to those reported by Narahashi et al (1976) for 2-AP on squid axon. In *Myxicola* axons 1 mmole/l 2-AP reduces the maximum potassium conductance by approximately 70%, and shifts the threshold for delayed rectification by 20 mV in the depolarizing direction. The time course of activation is not affected and the level of block is not influenced by strong depolarizing pulses or by repetitive pulses. There is therefore some similarity between the action of 2-AP on *Myxicola* axons and 4-AP^+ on muscle. Although Schauf et al do not make the point, their results can be analysed in terms of a tonic and voltage dependent block and described by an equation similar to equation 111.36: as shown in Table 9. From Fig. 3 of Schauf et al the action of 2-AP can be described by a tonic inhibition of 70% and a voltage dependent blocking reaction which has a dissociation constant which undergoes an e-fold change every 30 mV.

IV_{ii}•

Comparison of the Blocking Actions of 4-AP^+_i with those of TEA^+_i and Cs^+_i .

Comparison of the Blocking Actions of 4-AP_i^+ with those of TEA_i^+ and C_9^+ .

When TEA ions are present inside squid axon and frog node they selectively block the potassium current (Armstrong, 1966; Hille & Armstrong, 1972; Koppenhofer, 1968). The long chain derivative of TEA, non^utriethylammonium (C_9), is more potent than TEA; in addition the potassium current is not maintained in the presence of C_9 but instead inactivates in a qualitatively similar manner to the sodium current (Armstrong, 1969; Armstrong & Hille, 1972). It is of interest therefore to compare the mode of action of TEA_i^+ and the inactivation induced by its derivatives on nerve with the inactivation induced by 4-AP_i^+ on muscle. The comparable experiments with TEA^+ and C_9^+ inside skeletal muscle have not yet been done.

In squid axons bathed in isotonic potassium ringer Armstrong and Binstock (1965) have shown that the blocking action of TEA_i^+ depends on the direction of current flow. Outward current was completely abolished whilst inward current was little affected. Similarly Armstrong & Hille (1972) have shown that C_9 inactivation in frog node is also current dependent. In contrast, 4-AP_i^+ induced inactivation in muscle proceeds in the absence of net membrane current and independently of the direction of the current flow which suggests that the 4-AP block is a voltage dependent reaction.

The time course of inactivation induced by C_9^+ in nerve and 4-AP_i^+ in muscle is approximately exponential but only after an initial delay, (Armstrong, 1973). This delay has been interpreted to mean that the blocking ion must wait until the channels open before it can gain access to the blocking site, (Armstrong, 1969; Armstrong & Hille, 1972). The activation mechanism or 'gate' which controls the opening of the channel may therefore be located between the inner surface of the membrane and the blocking site, S_i^+ . The initial delay observed in the 4-AP_i^+ induced inactivation suggests that the activation gate is located at a similar site in muscle as it is in nerve.

When TEA ions are present inside a hyperpolarized frog node and the

node left unstimulated during the application, it is found that the delayed current is reduced only after the channel has been activated several times (Armstrong & Hille, 1972). This 'use dependent' blockage is similar to that attributed to 4-AP^+_1 in slowing the repolarizing phase of the action potential. This result also supports the idea that the activation gate in frog node and skeletal muscle is located near the inner surface of the membrane. Use dependent blockages of sodium channels have also been reported on myelinated nerve by Courtney (1974) with a derivative of lidocaine and by Marquis & Mautner (1974) on squid axon with sulphydryl reagents. In each case it is thought that the blocking site is only accessible to the chemical agent whilst the channels are open; in the closed state the gating mechanisms denies the blocking molecule ^{open} to the blocking site.

The potency of the TEA derivatives increases as the length of the side chain is increased (Armstrong, 1974). As the chain length increases the lipid solubility of that part of the molecule increases, which suggest that the binding site for the blocking molecule is hydrophobic (Armstrong, 1969, 1974, 1975). The 4-AP^+_1 cation also has a lypophylic 'tail' and may react with a similar lypophylic site near the inner mouth of the potassium channel in muscle.

In squid nerve the inactivation induced by C_9 proceeds at a rate approximately 10 times faster than the inactivation induced by 4-AP in muscle. The reason for this difference is not clear. It must be borne in mind that C_9 induced inactivation is current dependent whereas 4-AP inactivation appears to be voltage dependent. Also the possibilities must be considered that there are differences in the lypophylic nature of the

inner aspects of the channels in the different tissues and that there may be differences in the interaction of the hetroaromatic aminopyridine and the aliphatic C₉ chain with the lipid.

The conductance of a single channel can be estimated from the rate of the induced inactivation (Armstrong, 1974) using the expression:

$$\begin{aligned} (\text{g})_{\text{single channel}} &= \bar{g}_K (K^+_{\text{entry rate}}) \cdot \left(\frac{C}{V}\right)^{-1} \\ &= \bar{g}_K (X^+_{\text{entry rate}}) \cdot \frac{C}{V} \cdot \frac{K^+_i}{X^+_i} \cdot \gamma^{-1} \end{aligned}$$

where C is the charge on a single positive ion; V is the applied voltage; and γ is proportionally constant which expresses the relative ease of entry of the blocking ion into the channel compared to the potassium ion. If γ is assumed to be unity then in squid axons the conductance of a single channel assessed from C₉ inactivation is approximately 3×10^{-12} mho. In skeletal muscle if the same assumptions are made the rate of induced inactivation by 4-AP⁺_i gives a figure for the single channel conductance of 2×10^{-14} mho. The best estimate of the single channel conductance is probably 10^{-11} mho, calculated using membrane noise analysis (Siebenga, 1973). The estimates in squid axon and skeletal muscle are underestimates probably because γ is not unity. This is to be expected as a result of the dragging effect of the lypophylic tail of each molecule in the channel wall. It is interesting to note that the single channel conductance estimated using 4-AP⁺_i inactivation is of the same order of magnitude as the conductance expected from a carrier molecule such as non-actin (Armstrong, 1974; Lauger, 1972). The diameter of the lipid soluble non-actin molecule is approximately 5 Å and therefore similar in size and nature to lypophylic tail of the 4-AP cation. The similarity in the rates of movement within the membrane suggests that they interact with the membrane in similar ways. This would tend to support the idea that part of the 4-AP molecule is embedded in a lipid

phase in the membrane in muscle.

This apparent interaction of the lypophylic tail of internally present 4-AP with the channel wall is in contrast to the absence of any such interaction of 4-AP present externally. This situation tends to suggest that the structure of the channel wall in muscle is different at its outer and inner aspects.

IV.iii. Comparison of the Blocking Actions of 4-AP⁺_o and TEA⁺_o.

Comparison of the Blocking Actions of 4-AP^+_o and TEA^+_o .

It is also of some interest also to compare the blocking action of 4-AP^+_o and externally applied TEA^+_o ions on the potassium channels of nerve and muscle. TEA^+_o has been shown to block selectively the delayed potassium current in frog node of Ranvier (Hille, 1967; Koppenhöfer, 1967), and in polarized muscle fibres bathed in hypertonic solution (Stanfield, 1970a). TEA^+_o has no effect on the potassium currents in squid axons (Armstrong et al, 1965). Table 10 compiles the results obtained in each tissue. Both TEA^+_o and 4-AP^+_o are selective and effective in reducing the potassium current. In each tissue the dose response relationship suggests that one molecule binds to a single blocking site; the dissociation constant for each reaction is shown in row 1. In muscle 4-AP is more potent than TEA, although the difference is not as great as it is in nerve, where 4-AP is 2000 times more potent than TEA, (Pichon & Meves, 1975).

In polarized skeletal muscle the effectiveness of TEA is not influenced by the membrane potential (Stanfield, 1970), and resembles the tonic block exerted by 4-AP^+_o . It is possible that the same site is involved during the TEA^+_o block and the 4-AP^+_o tonic block. Although there is no direct evidence for this at present, the situation could be tested easily by determining whether 4-AP^+_o and TEA^+_o compete with each other in blocking the channel.

In muscle, the phasic block induced by 4-AP^+_o probably takes place at a site approximately $2/3$ of the way across the membrane from the outside. This site is apparently inaccessible to TEA, perhaps due to the larger dimensions of the TEA molecule. If the dimensions of the TEA and 4-AP molecules are 8 \AA and $6 \times 4 \text{ \AA}$ respectively (Armstrong, 1974; Christen et al, 1975), then the outer portion of the channel must have dimensions between 6 and 8 \AA .

In frog node, Hille, 1967; Koppenhöfer, 1967, found that the level of

TABLE 10

COMPARISON OF THE BLOCKING ACTIONS OF TEA_o⁺ and 4-AP_o⁺

	<u>FROG NODE</u>		<u>SKELETAL MUSCLE</u>		
	Hille (1967) TEA _o ⁺	Koppenhofer et al (1967) TEA _o ⁺	Stanfield (1970) TEA _o ⁺	Loc Cit TEA _o ⁺	4-AP _o ⁺
K _{diss}	0.4 mM	-	8 mM	~5 mM	1 mM
Voltage dependence of block	-	decrease on depolarization	none	-	decrease on depolarization
•T _n	none	increase (70% by 0.3 mM)	increase (80% by 58 mM)	* none (5 mM)	* none (1 mM)
•T _k	-	-	none	none	none
Shift in Threshold	none	-	-5 mV	-	none

* Initially slowed by a factor of 10 by formaldehyde treatment (c.f. Argibay, 1974).

block by TEA was decreased upon strong depolarization. On the other hand Hille (1966), in the same tissue finds no voltage dependence. At present there is no apparent reason for the discrepancy, and the existence of one or two binding sites for TEA^+_o in frog node remains uncertain.

The time course of activation of the delayed current in frog node (Koppenhöfer, 1967) and in polarized fibres (Stanfield, 1970) is slowed by TEA^+_o . In the present experiments neither 4-AP^+_o nor TEA^+_o affect the time course of the activation. This is perhaps not surprising in view of the finding that formaldehyde treatment alone slows the rate of activation (Argibay, 1974). The time course of the inactivation mechanism per se is not affected by TEA^+_o or 4-AP^+_o .

The dependence of the voltage sensitive phase of 4-AP^+_o blockage on the degree of inactivation of the delayed potassium channel in muscle has been interpreted to mean that part of the inactivation mechanism, when in position, denies 4-AP^+_o access to its blocking site. A similar interpretation is presented to account for the 'use dependent' blockage of H^+_o and TEA^+_o ions. It is thus likely that the inactivation mechanism of the potassium channel in skeletal muscle is located near the outer surface of the membrane. In addition the finding that the rate of inactivation is independent of membrane potential also suggest that the mechanism is outwith the electric field, and on the surface of the membrane. It is interesting to note that the inactivation mechanism of the sodium channels in nerve is thought to be located near the inner surface, (Armstrong et al, 1973; Strichartz, 1973; & Courtney, 1974).

IV_{iv}

A Model for the Potassium Channel in Skeletal Muscle

A Model for the Potassium Channel in Skeletal Muscle

It is proposed that the blocking actions of 4-aminopyridine on the delayed potassium channel in skeletal muscle can be accounted for by an action of the cationic form of the molecule present in the extracellular solution and the cationic form inside the cell. The cationic form in the external solution, 4-AP^+_o , causes a voltage independent block in addition to a voltage dependent block which increases as the internal potential is made more negative. The internally present cation induces a voltage dependent inactivation, the rate of which increases for more positive potentials and also depends on the concentration of 4-AP^+_i . There are also some indications regarding the nature of the walls of the channel and the location of the activation and inactivation mechanisms.

It is useful to draw a possible structure for the potassium channel based on the interactions of 4-AP with the channel, although an oversimplification it is worthwhile to do so. Such a picture can help in visualizing how 4-AP and other agents may act and further can be used as a basis for the design of future experiments.

In Fig. 63 the potassium channel is represented as a long narrow pore in which there are at least four binding sites. These sites, S_i , S'_i , S'_o and S_o are inferred because they can be occupied by 4-AP^+ ; but in the "normal" state such sites would be occupied by K^+ ions in their passage through the channel. Between sites, S'_o and S'_i the structure of the channel is such that 4-AP ions do not pass whilst K^+ may; consequently this section may be regarded as the more selective portion of the channel. If this section of the channel in muscle is similar in diameter to that in nerve then the channel at this point is approximately 3 \AA , (Hille, 1975).

Site S_o lies on the outer surface of the channel. This site can accommodate TEA^+ (as suggested by the results of Stanfield, 1970) and 4-AP^+_o . Since TEA^+ does not apparently enter the channel from the outside whilst 4-AP^+_o does this suggests that this outer section has a diameter

INSIDE

MEMBRANE

OUTSIDE

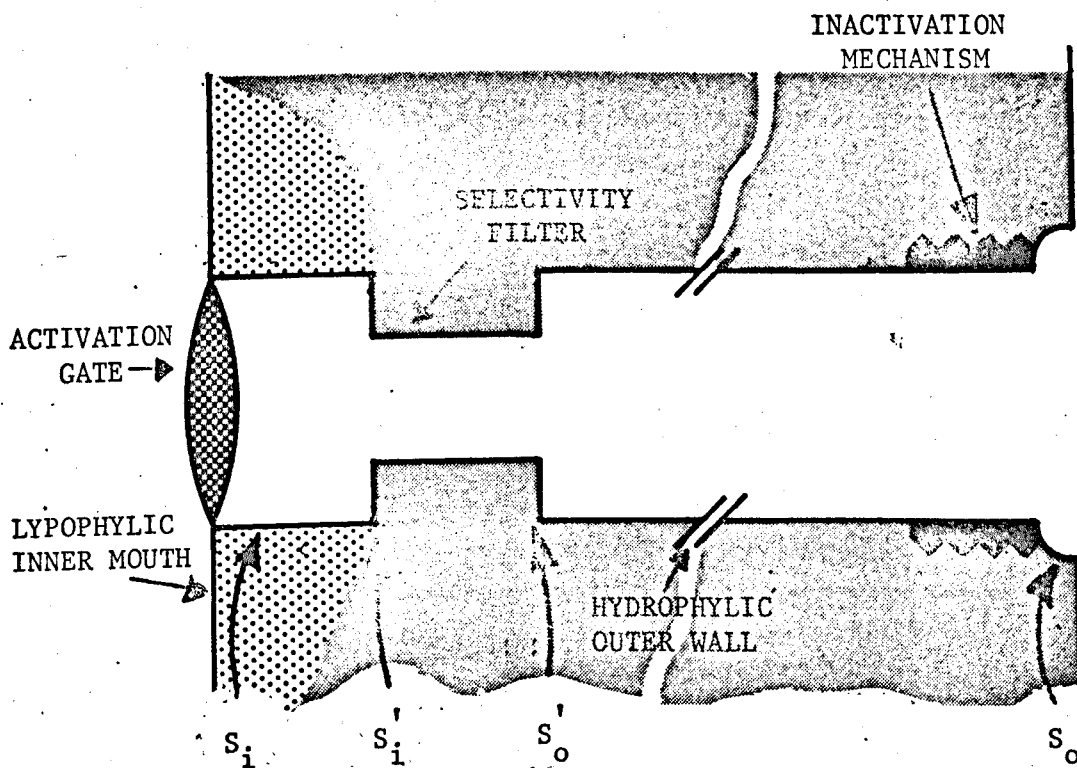
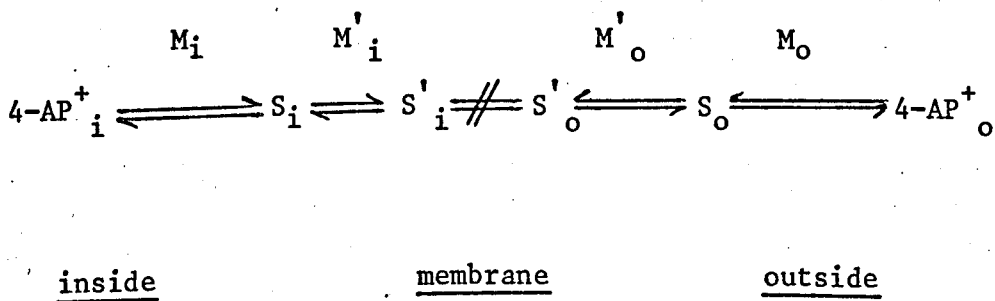


Fig. 63

between 6 - 8 Å (see page 96). The channel wall in this section appears to be hydrophylic. The evidence for this being the rapid movement of 4-AP^+_o in and out of the channel. Also, this section of the channel is a likely location for the inactivation mechanism; since the degree of inactivation was found to influence the 4-AP^+_o phasic block.

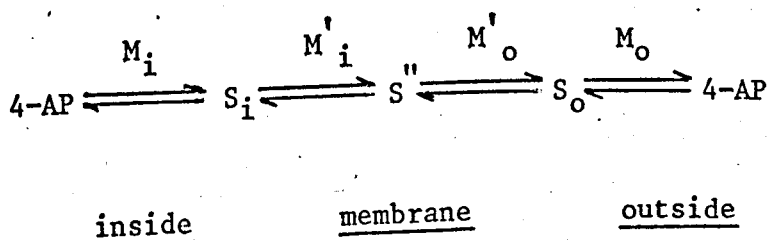
Near the inner aspect of the channel the wall is probably more lypophylic. This conclusion is based in the slow rate at which 4-AP^+_i can move in the electric field. The activation mechanism may be located in this section on the basis that 4-AP^+_i induced inactivation can only proceed in open channels.

In a more general way the channel and the blocking actions of 4-AP can be represented by the following:-



where M_i and M'_i describe the binding of 4-AP^+_i to sites S_i and S'_i ; movement of 4-AP from S_i to S'_i represents the voltage dependent induced inactivation; M'_o and M_o are the mechanisms which describe the voltage dependent and voltage independent blocking actions of 4-AP^+_o respectively at sites S'_o and S_o .

In this model of the potassium channel in skeletal muscle it is assumed that S'_i and S'_o are distinct sites, but the possibility must be considered that there is only one blocking site within the channel, S'' , thus:



However, there is some evidence which argues that there are two distinct blocking sites within the membrane. First, the voltage dependence of the dissociation constants which describe the blocking actions by mechanisms M'_i and M'_o are different. If this voltage dependence depends on the fraction of the applied electric field experienced by the site, and if the field within the membrane is linear then this suggests that the sites have different locations within the channel. Secondly, if S'_i and S'_o were the same site then it should in principle be possible for 4-AP to pass through the channel, but this does not occur, as may be concluded from the following observations. For example, if a single site were involved and it was occupied by 4-AP^+_o by mechanism M'_o , then under the appropriate conditions 4-AP^+_o may leave the site by the reverse of mechanism M'_i , and thereby pass through the channel. It would then follow that 4-AP^+ added exclusively to the extracellular solution (i.e. at $\text{pH}_o = 5.0$), would eventually become involved in mechanism M'_i . The experimental results suggest that this does not happen; the blockage produced by 4-AP^+_o in acid solution is mainly by mechanism M'_o and not M'_o and M'_i . A single blocking site within the potassium channel in nerve however is sufficient to account for the blocking actions in that tissue.

IV. A 5 Barrier Model

A 5 Barrier Model

The rate at which ions pass through such an open channel may be described using Eyring rate theory, in terms of elementary jumps over a series of energy barriers. The progress of an ion over each energy barrier is proportional to the number of molecules that attain the energy needed to form the activated complex or transition state. Such an energy profile for the potassium channel is shown below.

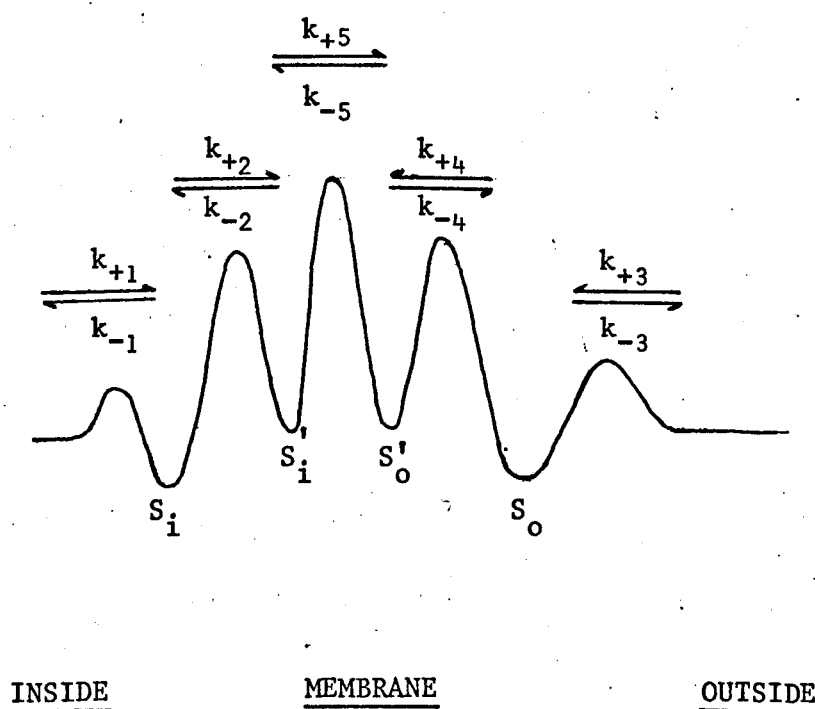


FIG. 64.

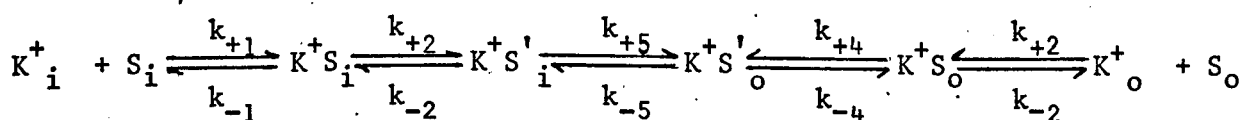
The rate constant for the passage of an ion over each barrier is related to the standard Gibbs free energy of activation ΔG_i , by the expression:

$$k_i = K_i \left(\frac{kT}{h} \right) \exp \left(-\frac{\Delta G_i}{RT} \right)$$

where K_i is the transmission co-efficient and has a value close to unity (see Hille, 1975a). In the presence of an external electric field the Gibbs free energy term includes a term for the potential as well as chemical energy, (e.g. Strichartz, 1973). The four binding sites are represented by energy minima and give rise to five energy barriers or

peaks over which an ion must cross in order to pass through the channel. For potassium ions these barriers may incorporate the loss of part of its hydration shell. The highest barrier is positioned between S'_o and S'_i , and may be taken to represent the selectivity filter. For potassium ions this barrier is relatively low, consequently there is little hindrance to the movement of K^+ , however, for 4-AP⁺, and other ions the barrier is high; 4-AP cannot pass through and remains lodged within the channel, at sites S'_o and S'_i causing a blockage.

If only one ion is allowed into the channel at a time then the four site model can be represented by the following kinetic scheme:



where the permeant ion undergoes five sequential transitions over the 5 energy barriers. However, there is some evidence that potassium ions interact with each other in transit through the potassium channel in nerve (Hodgkin & Keynes, 1955) and possibly also in muscle (Keynes, 1954; Argibay, 1974). This suggests that more than one ion can be present within the pore at any one time. In this situation the height of any energy barrier will also depend on whether the adjacent site is occupied or unoccupied. Such a channel has been called a 'single file pore', (Hodgkin & Huxley, 1952d; Hille, 1975a).

The kinetic scheme for a four site channel which can be occupied by more than one ion is shown in Fig. 65. With this scheme a maximum of four ions may be within the channel at any one time: one in each of the energy minima; ions can only enter the channel via the outer or inner sites and cannot pass each other within the channel. There are 15 possible occupied states and one unoccupied state in the model and there is perpetual transition between the possible states. All the allowed transitions are shown as interconnecting lines, each line representing a forward and a reverse reaction.

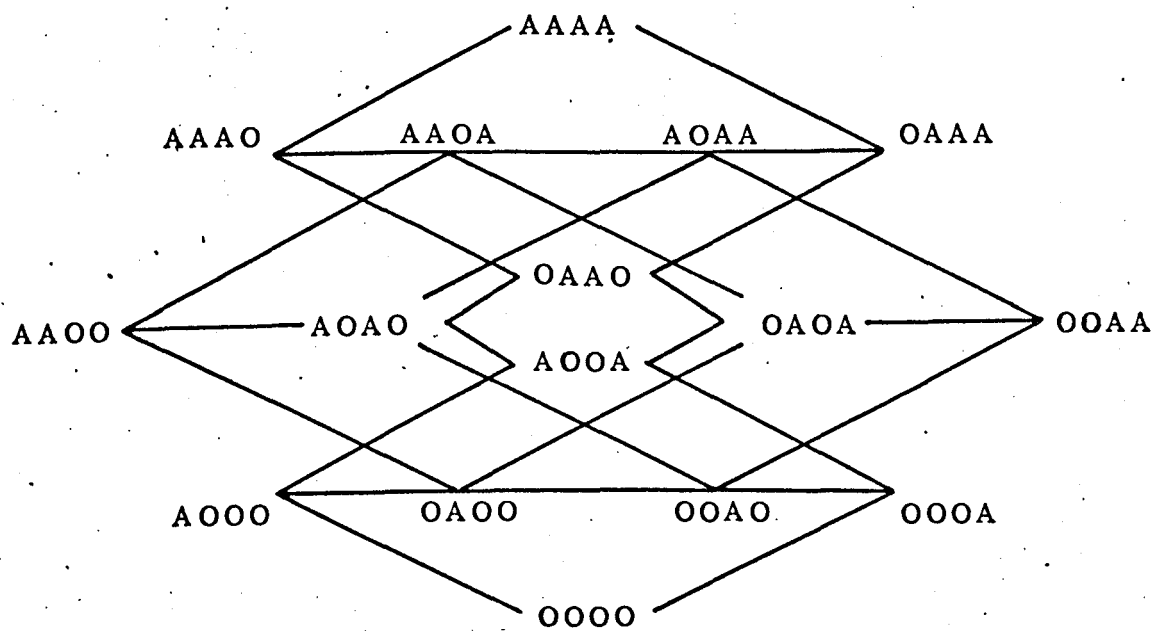


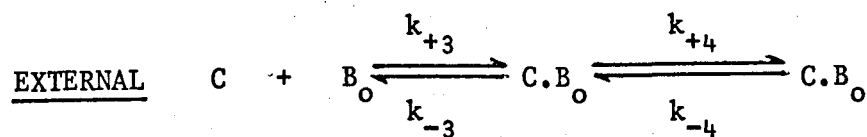
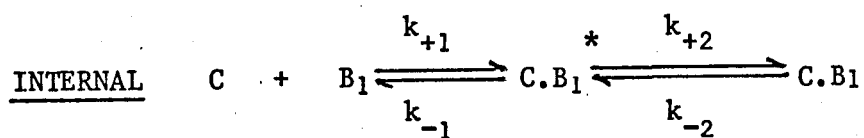
Fig. 65

Hille (1975a) has used the same approach to describe a permeability model for the sodium channel in frog node of Ranvier. In this model there are three sequential binding reactions for Na^+ ions but only one ion is admitted into the channel at any one time. The kinetic scheme for such a sodium pore is simpler than that described for the potassium pore. Hille (1975b) has been able to solve his kinetic model numerically, and to account for many of the experimentally observed properties of the more studied sodium channel.

Recently, Sandblomb, Eisenman and Neher (1977) have analysed the conducting properties of Gramacidin A channels in terms of a model which has 4 binding sites within the channel and where the channel may be multiply occupied. This situation is similar to that outlined for the potassium channel in muscle in this thesis. At the present time it is impossible to solve the model outlined here or to test the Sandblomb, Eisenman and Neher model on the delayed potassium channel in skeletal muscle due to lack of the appropriate experimental results. The solution of this model in terms of individual rate constants and binding constants must await more experimental information on the saturation, selectivity and block of the delayed potassium channel in skeletal muscle.

It is possible to represent the blocking actions of 4-AP in an open channel using a kinetic scheme similar to that shown in Fig. 61. The simplest situation is when 4-AP interacts with an empty pore; this is diagrammatically shown in Fig. 66(a). It is assumed that 4-AP cannot pass through the channel, consequently, the transition between the inner two sites S'_0 and S'_i is not allowed. The channel is blocked when either of the two sites S_0 and S'_0 are occupied or when the inner site S'_i is occupied. Thus when considering the fraction of channels blocked it is only necessary to consider the singly occupied channels; the reaction scheme simplifies to that shown in Fig. 66(b). This scheme may be separated into an inner and an outer mechanism and each written separately

as shown below:



where the * denotes a channel occupied by the blocking ion but not blocked.

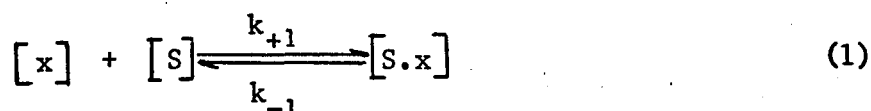
The reaction schemes have been written out with their individual forward and reverse rate constants; these schemes are also derived in the results section for which values for the rate constants have been calculated.

V. APPENDIX

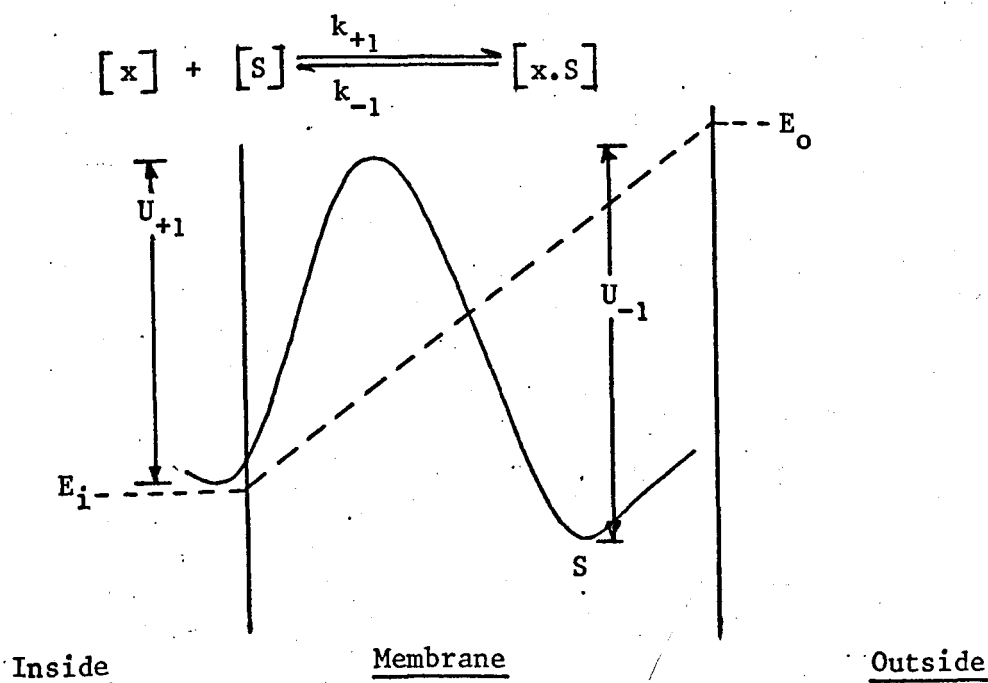
APPENDIX

To account for the influence of membrane potential on the blocking actions of the aminopyridines, a model for voltage dependent equilibria is discussed below. The model presented here is similar to those reported by Strichartz (1973), Woodhull (1972) and Hagiwara (1976).

Consider the case of a blocking ion linking to a site within a channel in the absence of any electric field, i.e.



where x is the concentration of the blocking ion; S the concentration of unblocked channels; $S.x$ the concentration of blocked channels; and k_{+1} and k_{-1} the forward and reverse rate constants respectively. A convenient way of representing such a scheme is to draw its "energy profile", as shown below.



Such an energy profile shows the energy changes of an ion as it is moved from outside the membrane, to a site, S , inside. To gain access to this site the ion must 'jump over' an energy barrier U_{+1} , and to leave the site must return over the barrier U_{-1} . Such barrier may incorporate electrostatic

interactions or the loss of waters of hydration. The rate at which the forward and reverse transitions proceed is given by:-

$$k_{+1} = K_{+1} \left(\frac{kT}{h}\right) \exp\left(\frac{-U_{+1}}{RT}\right) \quad (2)$$

$$k_{-1} = K_{-1} \left(\frac{kT}{h}\right) \exp\left(\frac{-U_{-1}}{RT}\right) \quad (3)$$

where R, T, k and h have their usual meanings; and K_i is a transmission co-efficient, (see Hille, 1975b).

Consider now, the effect of applying an electric field across the membrane. For simplicity, it is assumed that the field varies linearly across the membrane. The ease with which the ion enters and leaves the blocking site will now be altered, and the forward and reverse rate constants will now contain an electrical term (see Sandblomb et al, 1977).

Thus:-

$$k_{+1} = K_{+1} \left(\frac{kT}{h}\right) \exp\left(\frac{-U_{+1}}{RT} - \frac{zFE\delta}{2RT}\right) \quad (4)$$

$$k_{-1} = K_{-1} \left(\frac{kT}{h}\right) \exp\left(\frac{-U_{-1}}{RT} + \frac{zFE\delta}{2RT}\right) \quad (5)$$

where z is the valency of the blocking ion and δ is the fraction of the applied electric field experienced by the site S. In the case considered here, δ will depend on the location of the site within the membrane.

Equations (4) and (5) may be simplified to become

$$k_{+1} = C_{+1} \exp\left(\frac{-zFE\delta}{2RT}\right) \quad (6)$$

$$k_{-1} = C_{-1} \exp\left(\frac{zFE\delta}{2RT}\right) \quad (7)$$

where $C_{+1} = K_{+1} \left(\frac{kT}{h}\right) \exp\left(\frac{-U_{+1}}{RT}\right)$ and $C_{-1} = K_{-1} \left(\frac{kT}{h}\right) \exp\left(\frac{-U_{-1}}{RT}\right)$.

The dissociation constant for the reaction is thus given by

$$\frac{k_{-1}}{k_{+1}} = K_0 \exp \left(\frac{zFE\delta}{RT} \right) \quad (8)$$

where K_0 is the dissociation constant in the absence of any electric field.

From equation (1) and (8);

$$\frac{[S.x]}{[S][x]} = C_0 \exp \left(\frac{zFE\delta}{RT} \right) \quad (9)$$

An expression for the fraction of channels not blocked can then be derived from equation (9).

If S_T is the total number of available channels and

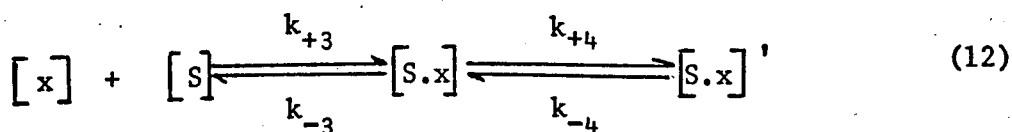
$$[S_T] = [S] + [S.x] \quad (10)$$

then the fraction of channels not blocked at any membrane potential (E) is given by

$$\frac{[S]}{[S_T]} = (1 + [x] C_0 \exp \left(\frac{zFE\delta}{RT} \right))^{-1} \quad (11)$$

The fraction of channels blocked by 4-AP_o^+ and 4-AP_i^+ as determined experimentally cannot be fitted with such a simple equation as equation (11). It is necessary therefore to modify the model to account for such differences.

Consider the blocking actions of 4-AP_o^+ which may be separated into a voltage dependent block in addition to a voltage independent block. A reaction scheme which can describe such a situation is shown below.



Consider first the special case where binding reaction, with rate constants k_{+3} and k_{-3} , is not influenced by potential; while the second reaction is voltage dependent. By using equation (12) and by applying similar assumptions to those used to derive equation (11) the fraction of channels not blocked is given by:-

$$\frac{[S]}{[S_T]} = (1 + [4-AP] \left(\frac{k_{-3}}{k_{+3}} + \frac{k_{-3}}{k_{+3}} C_3 \exp \left(\frac{ZF E \delta}{RT} \right) \right)^{-1} \quad (13)$$

This formulation implies that $4-AP^+$ binds in a dose dependent manner to a site near the channel but outside the electric field. When in this position 4-AP blocks the channel. The remaining unblocked channels are susceptible to block by the other voltage dependent reaction. This second reaction must be at a different site which lies within the electric field. Equation (13) is adequate to account for the steady state blocking actions of $4-AP^+$.

For the more general case, when both reactions are influenced by the membrane potential then the fraction of channels not blocked is given by:

$$\frac{[S]}{[S_T]} = (1 + [4-AP] \left(C_3 \exp \left(\frac{ZF E \delta_3}{RT} \right) + C_3 C_4 \exp \left(\frac{ZF E (\delta_3 + \delta_4)}{RT} \right) \right)^{-1} \quad (14)$$

Note that the overall voltage dependence of the second term in equation (14) is $(\delta_3 + \delta_4)$; and consequently may have a value between 0 and 2.

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